Molecular Analysis of Sexual Sporulation in *Aspergillus nidulans*

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Hiermit versichere ich, daß ich die vorliegende Arbeit “Molecular Analysis of Sexual Sporulation in *Aspergillus nidulans*“ selbständig verfaßt, keine anderen als die angegebenen Hilfsmittel verwendet und sämtliche Stellen, die im Wortlaut oder dem Sinn nach anderen Werken entnommen sind, mit Quellenangaben kenntlich gemacht habe.

Marburg, im März 2003

Im Zusammenhang mit der Thematik der vorliegenden Dissertation wurden bzw. werden folgende Publikationen erstellt:

Wei, H., Scherer, M., Singh, A., Liese, R. and Fischer, R. (2001). *Aspergillus nidulans* α-1,3 glucanase (mutanase), *mut*A, is expressed during sexual development and mobilizes mutan. *Fungal Genetics and Biology.* 34(3), 217-227. (with Cover).

Scherer, M., Wei, H. and Fischer, R. (2002). *Aspergillus nidulans* catalase-peroxidase, CpeA, is upregulated during sexual development through the APSES transcription factor StuA. *Eukaryotic Cell.* 1(5), 725-735.

Wei, H., Requena, N. and Fischer, R. (2003). The MAPKK-kinase SteC regulates conidiophore morphology and is essential for hyphal fusion and sexual development in the homothallic fungus *Aspergillus nidulans. Molecular Microbiology.* 47(6), 1577-1588.

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Abbreviations

APS	ammonium perooxdisulfate
CM	complete Medium
DAPI	4,6-diamidino-2-phenylindol
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonat
DTT	1,4 Dithiothreitol
GFP	green fluorecent protein
IPTG	isopropylthio-β-D-galactoside
LacZ	β-galactosidase
LB	Luria-Bertani medium
MM	minimal medium
RT-PCR	reverse transcriptase-polymerase chain reaction
SAP	shrimp alkaline phosphatase
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylendiamine
X-Gal	5-bromo-4-chloro-3-indoxyl-β-D-galctoside

Content

1 Summary

Aspergillus nidulans is a filamentous fungus, able to reproduce with mitotically derived conidiospores (conidia) and meiotic ascospores. Both spore forms are generated at or in morphologically differentiated structures called conidiophores and cleistothecia (fruiting bodies), respectively. Whereas the developmental program of conidiophore formation is well studied, cleistothecium differentiation is only poorly understood. *A. nidulans* is especially attractive to analyze fruiting body formation because it is a homothallic fungus and does not require a mating partner to initiate the developmental program.

A subtractive cDNA library of *A. nidulans* was established in previous work to identify differentially expressed genes during sexual development. Two clones from this substractive hybridisation library (SSH) were further studied in this work. One displayed homology to fungal α-1,3 glucanases (mutanase). Since α-1,3 glucan was considered as main reserve material accumulated during vegetative growth as a cell wall component and consumed during sexual development for the formation of cleistothecia, this gene was analysed in detail. The gene, *mutA*, is disrupted by 3 introns and encodes a putative protein of 48 kDa molecular mass with a signal peptide for secretion at the N-terminus. The deduced protein displays 24-42 % identical amino acids to mutanases of other fungi. A proposed mutan binding domain characterised in e.g. *Penicillium* is not present in *A. nidulans*. Expression analysis of the *mutA* promoter fused with *sgfp* revealed specific induction of the gene during sexual development in Hülle cells. To study the role of *mutA* during sexual differentiation, a *mutA* deletion strain was constructed. Although degradation of mutan was affected in this strain, it was still able to form cleistothecia with a similar number as wild type. These results suggest that additional carbon sources are available during sexual development. In minimal medium supplemented with mutan fraction as a sole carbon source, *mutA* wild type and overexpression strains showed much denser growth in contrast to a sparse growth of *mutA* mutant strains. The progressive 5' deletion of the *mutA* promoter fused with *sgfp* led to identify three putative DNA-binding regions for regulatory factors. One of those potential factors was characterized as a 40 kDa protein. Mass-spetrometric analysis remains to be done to identify this protein.

Another gene found in the SSH library showed high homology to high-affinity hexose transporter genes. The gene, *hgtA*, encodes an open reading frame of 2 kb interrupted by 6 introns

with 44-67 bp in length. The translated sequence of 531 amino acids has a molecular mass of 59 kDa. Hydrophobicity values showed the presence of 12 putative transmembrane (TM) domains, a characteristic feature of the major facilitator superfamily. The HgtA protein displays 32-42 % identical amino acids to other glucose transporter genes of other fungi. *ΔhgtA* strains show no evident phenotype compared with a wild type strain. The expression of *sgfp* fused with the *hgtA* promoter revealed a specific induction of *hgtA* in ascogenous hyphae within cleistothecia.

In addition to the investigation of the target genes that are involved in sexual development of *A. nidulans,* one upstream factor was also studied. Extracellular signals can be transduced into intracellular responses by the action of MAP kinase cascades. Sequential phosphorylation results in the transient activation of a MAP kinase, which in turn activates certain transcription factors and thus a set of pathway-specific genes. Many steps in this cascade are conserved, and homologues have been discovered from yeast to human. The present work has characterized the MAPKK kinase, SteC, a homologue of *Saccharomyces cerevisiae* Ste11, in *A. nidulans*. The 886-amino-acid-long protein shares the highest similarity to *Neurospora crassa* Nrc-1. Deletion of the gene in *A. nidulans* results in a slower growth rate, the formation of more branched hyphae, altered conidiophore morphology, an inhibition of heterokaryon formation and a block of cleistothecium development. The gene is transcriptionally activated during asexual development and controls the phosphorylation of two putative kinases.

Zusammenfassung

Aspergillus nidulans ist ein filamentöser Pilz, der sich mit mitotischen Conidiosporen (Conidien) und meiotischen Ascosporen vermehren kann. Beide Sporenformen werden an oder in morphologisch differenzierten Strukturen, den Conidiophoren bzw. den Cleistothecien (Fruchtkörper) gebildet. Während das Entwicklungsprogramm der Coniophorbildung sehr gut untersucht ist, wird die Cleistothecienbildung noch nicht gut verstanden. *A. nidulans* ist zur Untersuchung der Fruchtkörperbildung besonders gut geeignet, da er ein homothallischer Pilz ist, der keinen Kreuzungspartner zur Einleitung des Entwicklungsprogramms benötigt.

In einer vorangegangenen Arbeit wurde eine subtraktive cDNA Genbank etabliert, um während der sexuellen Entwicklung differenziell exprimierte Gene zu identifizieren. In der vorliegenden Arbeit wurden zwei der Klone der subtraktiven Genbank (SSH) molekular untersucht. Einer der Klone wies Homologie zu α-1,3 Glucanasen (Mutanase) auf. Da α-1,3 Glucan während des vegetativen Wachstums als Reservematerial angehäuft wird, um dann während der sexuellen Entwicklung zur Bildung der Cleistothecien verwendet zu werden, wurde dieses Gen im Detail analysiert. Das Gen, *mutA*, wird durch 3 Introns unterbrochen und kodiert für ein putatives Protein mit einer berechneten molekularen Masse von 48 kDa. Das abgeleitete Protein enthält am N-Terminus eine Signalsequenz, die auf eine Sekretion des Proteins hindeutet. MutA weist 24-42 % identische Aminosäuren im Vergleich zu Mutanasen in anderen Pilzen auf. Eine in anderen Enzymen vorgeschlagene Mutanbindedomäne, wie z.B. in *Penicillium*, ist in MutA nicht vorhanden. Expressionsanalyse mittels einer Fusion des *mutA* Promotors mit *sgfp* zeigte eine spezifische Induktion des Gens während der sexuellen Entwicklung in Hülle-Zellen. Um die Rolle von *mutA* während der sexuellen Differenzierung zu untersuchen, wurde ein *mutA*-Deletionsstamm hergestellt. Obwohl der Abbau von Mutan in diesem Stamm beeinträchtigt war, war der Stamm noch in der Lage, Cleistothecien mit einer ähnlichen Häufigkeit wie Wildtypstämme zu bilden. Das bedeutet, dass wahrscheinlich zusätzliche Kohlenstoffquellen während der sexuellen Entwicklung zur Verfügung stehen. In Minimalmedium, das mit isoliertem Mutan als einziger Kohlenstoffquelle supplementiert wurde, zeigten *mutA*-Wildtyp und Überexpressionsstämme dichteres Wachstum als die *mutA* Mutante. Progressive 5'-Deletionen des *mutA*-Promotors fusioniert mit *sgfp* führten zur Identifikation von drei möglichen DNA-Bindungsregionen für regulatorische Faktoren.

Einer dieser potenziellen Faktoren wurde als ein 40 kDa Protein charakterisiert. Eine massenspektroskopische Analyse zur Identifikation dieses Proteins wurde noch nicht durchgeführt.

Ein zweites Gen, das in der SSH-Genbank gefunden wurde, zeigte Homologie zu hoch-affinen Hexosetransportergenen. Das Gen, *hgtA*, kodiert einen offenen Leserahmen von 2 kb, der von 6 Introns mit 44-67 bp Länge, unterbrochen wird. Die translatierte Sequenz von 531 Aminosäuren besitzt eine molekulare Masse von 59 kDa. Hydrophobizitätsanalysen des Proteins ergaben das Vorhandensein von 12 möglichen Transmembrandomänen, was eine charakteristische Eigenschaft der "major facilitator family" ist. Das HgtA Protein wies 32-42 % identische Aminosäuren zu anderen Glucosetransportern anderer Pilze auf. *ΔhgtA*-Stämme zeigten keinen evidenten Phänotyp im Vergleich zu Wildtypstämmen. Durch Expression von *sgfp* unter der Kontrolle des *hgtA*-Promotors wurde eine spezifische Induktion von *hgtA* in ascogenen Hyphen innerhalb der Cleistothecien festgestellt.

Zusätzlich zur Untersuchung der beiden differenziell exprimierten Gene, wurde ein regulatorischer Faktor untersucht. Extrazelluläre Signale können durch MAP kinase Kaskaden in intrazelluläre Antworten übersetzt werden. Eine sequenzielle Phosphorylierung ergibt eine transiente Aktivierung einer MAP Kinase, die schliesslich bestimmte Transkriptionsfaktoren und damit stadienspezifische Gene in ihrer Aktivität reguliert. Die Signalkaskade ist evolutionär konserviert, so dass homologe Komponenten von der Hefe bis zum Menschen beschrieben wurden. In der vorliegenden Arbeit wurde die MAPKK Kinase, SteC, ein Homologes von Ste11 aus *Saccharomyces cerevisiae*, in *A. nidulans* untersucht. Das 886 Aminosäuren lange Protein zeigte die höchste Ähnlichkeit zu *Neruospora crassa* Nrc-1. Deletion des Gens in *A. nidulans* führte zu einer verringerten Wachstumsrate, der Bildung von stärker verzweigten Hyphen, einer veränderten Conidiophormorphologie, einer Hemmung der Heterokaryon- und der Cleistothecienbildung. Das Gen wird transkriptionell während der asexuellen Entwicklung induziert und reguliert die Phosphorylierung von mindestens zwei Kinasen.

摘 要

钩巢曲霉 (*Aspergillus nidulans*) 属于同宗配合的丝状真菌。它可有丝分裂产生无性的分生孢子 (condiospore, conidia) 和减数分裂产生有性的子囊孢子 (ascospore)。两种孢子分别由分生孢子梗 (condiophore) 和子囊壳 (cleistothecium) 产生。无性孢子的形成过程已有清晰的研究，但有性产孢的分子基础有较少的研究。由于钩巢曲霉为同宗配合的真菌，因而作为一个有性产孢子机制研究的模式种。该工作为项目“钩巢曲霉有性产孢的分子机制的研究”的一部分。

基于钩巢曲霉有性产孢的消减杂交文库 (SSH) 的建立。两个基因被进一步研究。第一个基因显示和其它真菌α-1,3 葡聚糖裂解酶 (α-1,3 glucanase; 木糖酶, mutanase) 高度的同源性。由于传统认为在钩巢曲霉无性产孢过程中菌丝累积大量 α-1,3 葡聚糖，而在后期 α-1,3 葡聚糖被降解重新利用以提供该菌有性产孢中子囊壳形成的碳源和能源。因而该基因被进一步研究。该基因转录为拥有 3 个内含子 1.5 kb 片段，编码一个 48 kDa 的酶，其 N-端信号肽序列表明该酶被分泌。该酶显示和其它真菌 α-1,3 葡聚糖裂解酶 24-42% 相同的氨基酸序列，但不同于青霉菌 (*Penicillium*) 和木霉菌 (*Trichodema*) 的 α-1,3 葡聚糖裂解酶，该酶缺乏一个预测的 C-端葡聚糖结合域。绿色荧光蛋白 (*sgfp*) 与 α-1,3 葡聚糖裂解酶启动子的融合揭示该酶特异性的在有性组织护卫细胞 (Hülle cells) 中的高效表达。为明确该酶的功能，一个 α-1,3 葡聚糖裂解酶缺失突变的菌系被转化获得，尽管在该突变菌系中α-1,3 葡聚糖降解被大大的影响，但仍形成和野生型菌系相似数量的子囊壳。因而表明在子囊壳发育中其它碳源被利用。仅用来源于 12 天生长的 α-1,3 葡聚糖裂解酶缺失突变菌系葡聚糖组分作为唯一碳源的培养基上， 野生型和 α-1,3 葡聚糖裂解酶超表达菌系显示浓密的生长， 而 α-1,3 葡聚糖裂解酶突变菌系显示非常稀疏的生长。 用绿色荧光蛋白作为一个报告基因，α-1,3 葡聚糖裂解酶启动子连续的 5’-端删除显示 3 个预测的调控蛋白结合区域。 调控蛋白的分离显示一个 40 kDa 调控蛋白结合在启动子 -1.7 kb 的位置。 另一个来源于消减杂交文库的基因显示和己糖转运蛋白 (hexose transporter) 高度的同源性。 该基因 (*hgtA*) 为 2 kb 有 6 个内含子的开放阅读框， 编码一个由 531 氨基酸组成的 59 kDa 的蛋白质。 疏水值预测该蛋白拥有 12 个垮膜区。 该蛋白显示和其它真菌葡萄糖转运蛋白 32-42 % 相同的氨基酸序列。 比较野生型菌系， 该基因缺失突变菌系未显示明显的表型差异。 绿色荧光蛋白与启动子的融合揭示该蛋白特异性的在子囊壳中子囊产生菌丝中 (ascogenous hyphae) 的高度表达。

除了与钩巢曲霉有性产孢的相关的目标基因的鉴定，上游与有性产孢信号转导相关的基因也被研究。细胞外信号可被丝裂原活化蛋白激酶 (mitigen-activated protein kinase, MAPK) 信号转导通路传递为胞内信号。依次的磷酸化导致一个 MAP 激酶的短暂激活，磷酸化 MAP 激酶激活调控蛋白， 因而导致特定的基因的表达。 在本工作中， 一个与 *S. cerevisiae* Ste11 同源的来源于钩巢曲霉激酶, SteC, 被进一步研究。该激酶由 886 氨基酸组成， 显示和脉孢菌 (*N. crassa*) 激酶高度的同源性。 该基因缺失突变菌系显示较慢的菌落生长速率，多分枝的菌丝，改变的分生孢子梗形态，正常异核形成的抑制以及子囊壳发育的封闭。该基因在无性产孢的早期被转录激活，调控至少两个激酶的磷酸化。

2 Introduction

Aspergillus nidulans is a filamentous, homothallic fungus belonging to the family of ascomycetes which is ubiquitously distributed worldwide. The scientific name is derived from two Latin words: *aspergillum* which is a device used to sprinkle holy water (as this resembles the asexual reproductive structure called a conidiophore) and *nidulans* which means nest-like (which refers to the sexual structure called a cleistothecium). Although its correct taxonomic name is *Emericella nidulans,* it is more widely known as *Aspergillus nidulans*. Other members of the ascomycete class include *Neuraspora crassa* (orange bread mould) and *Saccharomyces cerevisiae* (baker's yeast).

A. nidulans was established as genetic model organism in the 1950s (Pontecorvo et al., 1953). It has a relatively small, haploid genome of 28.5 Mb with about 8000 genes, spread over eight chromosomes with sizes ranging from 4.3-2.7 Mb. It is able to reproduce with mitotically derived conidiospores (conidia) and meiotic ascospores (Adams et al., 1998). Both spore forms are generated at or in morphologically differentiated structures called conidiophores and cleistothecia (fruiting bodies), respectively (Fischer, 2002)(Fig. 2.1). Whereas the developmental program of conidiophore formation is well studied, cleistothecium differentiation is only poorly understood. *A. nidulans* is especially attractive to analyze fruiting body formation because it is a homothallic fungus and does not require a mating partner to initiate the developmental program.

2.1 Sexual development and fruiting body formation in *A. nidulans*

A. nidulans is able to form fruiting bodies in the absence of a partner in a process called selfing, which is the development of cleistothecia in homokaryons, with two identical parent nuclei fusing and subsequently undergoing meiosis. This process results in meiospores with genotypes identical to the single parent nucleus. The *A. nidulans* mycelium can exist as homo- as well as a heterokaryon, the latter containing two genetically different sorts of nuclei after fusion of vegetative hyphae (Käfer, 1977; Upshall, 1981). In fungi with two different mating types, an *antheridium* cell ("male") fuses with an *ascogonium* ("famale") to give a dikaryotic hyphae. In the homothallic fungus *A. nidulans*, wild type sexual development is initiated either by mating of two strains or by selfing. Mating types are not known for this fungus, and no antheridium or

ascogonium structure can be observed. In analogy to other filamentous ascomycetes, it is assumed that an *A. nidulans* cell functionally equivalent to an ascogonium fuses to a second cell equivalent to an antheridium (Champe et al., 1994). Initiation of the sexual reproductive cycle and differentiation of three sexual tissue types take place shortly after conidiation begins (Champe, et al., 1994). The fused hyphae are surrounded by growing, unordered mycelium, which forms an increasingly packed "nest¨ and differentiates to form large thick-walled, multinucleated, globose Hülle cells (nurse cells), which develop by budding at the tips of specialized hyphae and form a tissue that envelops the young cleistothecium (Hermann et al., 1983).

The surrounding mycelium which forms the nest is subject to the formation of the cleistothecial primordium, a red-pigmented protective shell at maturity. In these nests, dikaryotic hyphae are formed by the fertilization events and subsequently undergo an extended series of coordinated cell and nuclear divisions. The developing cleistothecium grows out of the surrounding nest hyphae and Hülle cells, while the dikaryotic mycelium undergoes a switch from the coordinated nuclear and cellular division of ascogeneous hyphae to the formation of the so-called croziers (Fig. 2.2). Two nuclei are trapped in the topmost crozier cell by a series of divisions which require exact nuclear positioning and cell wall insertion. In every single crozier, a nuclear fusion event (karyogamy) forms a diploid nucleus ~70/80 h after spore germination (Pontecorvo, et al., 1953). This short zygote stage is immediately followed by meiosis, which results in four nuclei. After meiosis, one round of mitosis produces eight nuclei which are separated from each other by membranes. Another round of mitosis yields the eight binucleate ascospores organized in an octad of an *A. nidulans* ascus. Mature cleistothecia of wild type strains can reach a size of 200 µm and usually contain ~80,000 viable ascospores. The ascospores are red owing to the accumulation of a characteristic red pigment called asperthecin. Under laboratory conditions, cleistothecia and ascospores reach maturity ~100 h after the initial spore germination.

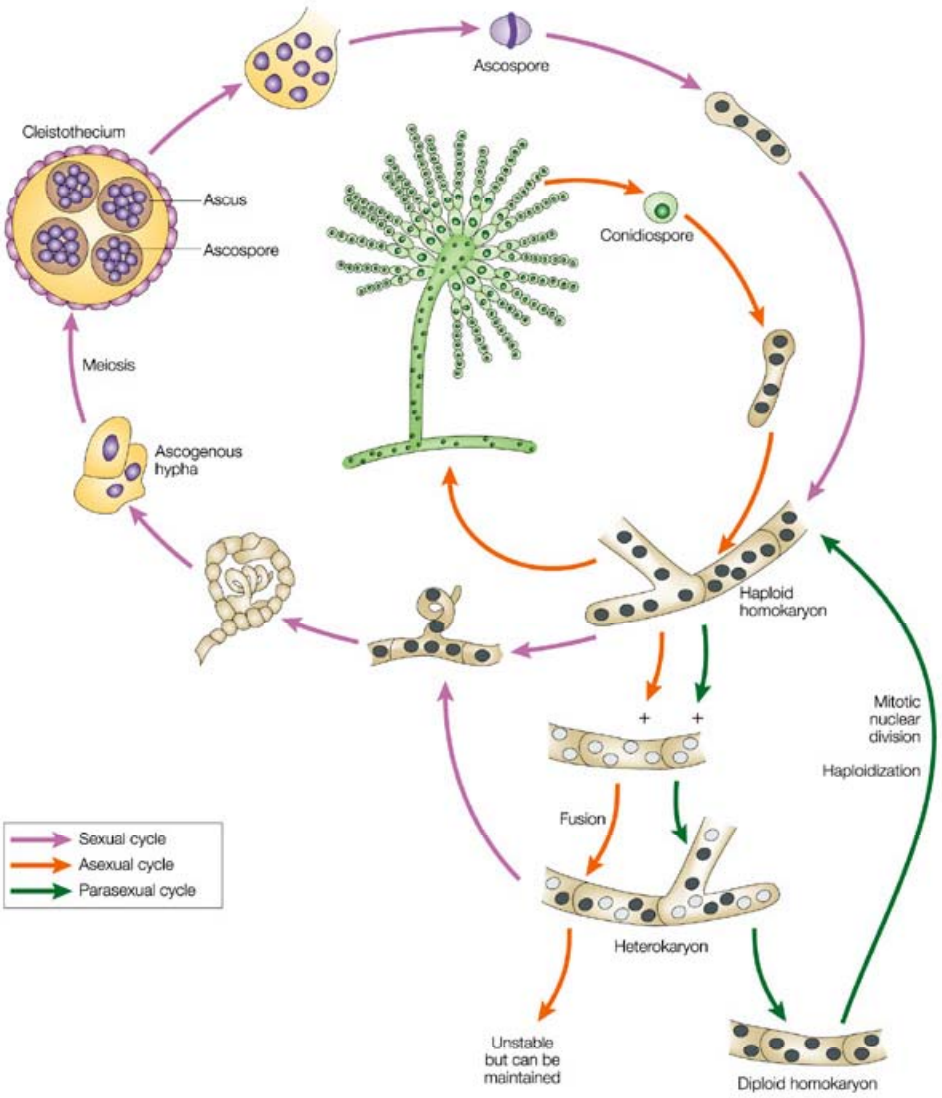


Fig. 2.1 Life cycle of *Aspergillus nidulans*. The fungal mycelium of *A. nidulans* is a web of branched filaments (hyphae) of connected compartments or cells, which each contain several nuclei (see centre figure). This mycelium, or homokaryon, which develops from a single haploid spore, differentiates many identical asexual spores known as conidia or conidiospores (see the asexual cycle in the figure). *A. nidulans* is homothallic, which means that it is self-fertile, but crosses can be initiated by hyphal fusions between homokaryons with genetically different nuclei (shown by white and dark green nuclei). The resulting heterokaryons are not stable, but can be forced to maintain a balanced ratio of the component nuclei by including complementing auxotrophic mutations in the parental nuclei and forcing growth without the corresponding supplements. *A. nidulans* can also reproduce sexually. In the fruiting body, which produces the sexual spores, a pair of nuclei that is destined for meiosis divides in synchrony to form a mass of cells known as the ascogenous hyphae. These hyphae are highly branched and each tip cell becomes an ascus (a specialized cell) in which the two haploid nuclei fuse. The diploid nucleus undergoes meiosis followed by a post-meiotic mitosis, which results in the formation of eight haploid ascospores. The fruiting body, called the cleistothecium, can hold tens of thousands of ascospores, which are released

into the environment when the cleistothecium bursts open. In addition to an asexual cycle and sexual cycle, a parasexual cycle offers the genetic benefits of meiosis achieved through a mitotic route (Pontecorvo & Kafer, 1958). The parasexual cycle is initiated when haploid nuclei fuse in the vegetative cells of a heterokaryon and continue to divide mitotically. Crossing over might occur between homologues and random chromosome loss restores the haploid chromosome number, which is eight in the case of *A. nidulans*. These events can be used to map gene orders and assign new genes to the eight linkage groups. Many closely related fungi of economic or medical importance, such as *A. niger*, *A. fumigatus*, *Fusarium oxysporum* and *Penicillium chrysogenum*, have no sexual cycle but are exploited experimentally or genetically using technologies developed for *A. nidulans* (Clutterbuck, 1992) (Taken from Casselton & Zolan (2002)).

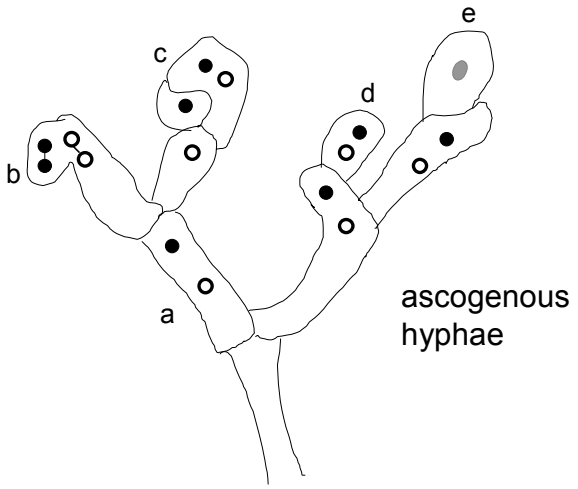


Fig. 2.2 illustration of crozier formation. In the ascogeneous, heterokaryotic hyphae (a) containing two haploid nuclei of opposite mating type, a hook-shaped structure is formed in which nuclei divide synchronously. (b) The penultimate, dikaryotic cell of the ascogonium forms the top crozier cell (c) in which, after fusion of the end cell and basal cell (d) karogamy (e) and further ascus development take place (Revised from Braus et al., 2002).

2.2 Determinants influencing fruiting body formation in *A. nidulans*

2.2.1. Environmental factors affecting sexual development

Environment factors such as light, CO_2, surface exposure, nutritional status, amino acids and hormones influence sexual development. Incubation of *A. nidulans* wild type in the dark for 24 h after inoculation leads to higher densities of fruiting bodies and less conidiation than during incubation in the light. In the dark, cleistothecia formation can also be initiated at an earlier time point than light-grown colonies (Zonneveld, 1977). *A. nidulans* wild type strains produce ~2000 fruiting bodies per cm^2 on petri dishes while developing approximately double the amount of cleistothecia and fewer conidiospores when air exchange is limited by taping the plates, which is

attributed to an increase of the CO_2 content. Usually, a mycelium does not differentiate in submerged culture but can be induced by transfer to solid medium. Sexual development requires a constant surface on which develop. A surface is indispensable for cleistothecia formation (Galbriath & Smith, 1968). Major nutritional compounds in the medium play a crucial role in sexual development. With reduced carbon source (0.8% glucose), fruiting body development was reduced or blocked compared to growth on 3% glucose (Han et al., 1994). Low nitrogen levels inhibit cleistothecia development (Zonneveld, 1975). A hormone was reported to influence sexual development, the so-called *psi* factor (precocious sexual inducer), an endogenous mixture of hydroxylinoleic acid moieties (Champe & El-Zayat, 1989; Champe et al., 1987). The *psi* factor extracted from growth medium of *A. nidulans* to a confluent plate culture strongly inhibits asexual sporulation and induces premature sexual sporulation.

2.2.2 Genetic determinants regulating fruiting body development

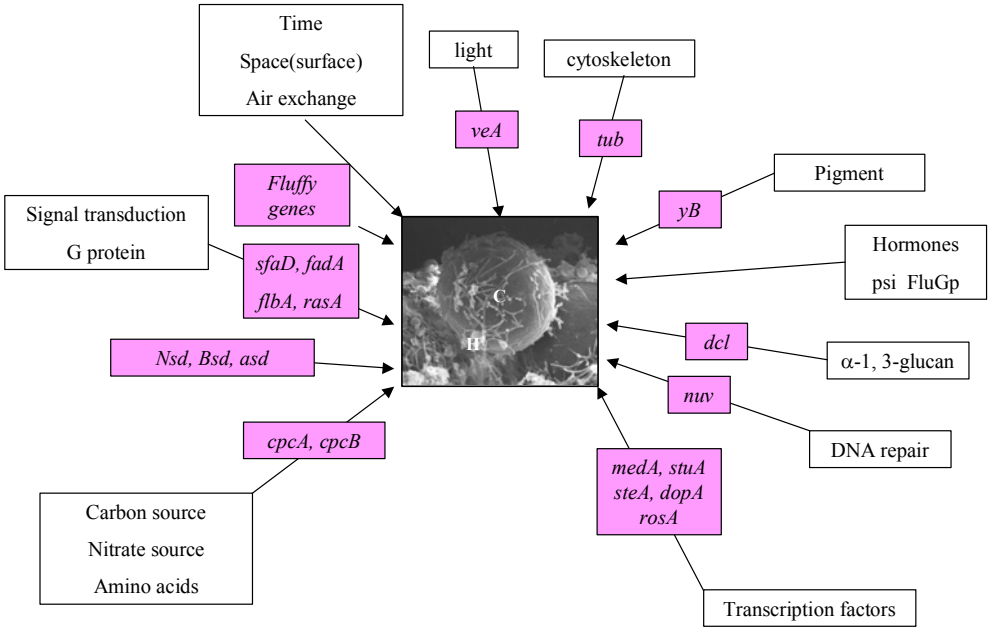
Perception of the environmental status as well as signal transduction relies on specific systems encoded by genetic determinants. From the ~8000 genes encoded by the *A. nidulans* genome, an estimated 6000 are required for "housekeeping" biochemical functions. A large proportion of the remainder of the genes is expected to be required for development and differentiation processes, e.g., the detection of environmental signals, signal transduction processes within developmental programs, or altered gene expression for coordinating the action of general and specialized biosynthetic enzymes.

The *veA* gene was identified as a gene responsible for a velvet-like phenotype (Käfer, 1965). The *veA1* mutation delays and reduces the development of sexual organs, resulting in the preferential development of asexual sporulation (Champe et al., 1981). Compared with the wild type (e.g. FGSC4), asexual development of the *veA1* mutant is much less affected by various environmental factors such as nutrients (Han et al., 1994a), light (Mooney & Yager, 1990) and temperature (Champe, et al., 1981). Therefore, it was proposed that *veA* acted as a negative regulator of asexual development and an activator of sexual development in response to the various environmental factors (Han, et al., 1994a; Timberlake, 1990). Protection of the culture plate from aeration and light inhibits asexual sporulation of the *veA*+ strains almost completely (Han et al., 1990; Mooney & Yager, 1990). The *veA* gene was recently cloned and its overexpression induced a larger number of sexual structure (Kim et al., 2002).

Although numerous signal transduction pathways are described and conserved among eukaryotes, the signalling compounds necessary for sexual development in *A. nidulans* are hardly known. This might be partally due to the fact that there is a spatiotemporal order of the two programs of asexual and sexual development subsequent to hyphal growth. However, both reproduction pathways seem to share signal transduction compounds. In addition, conidiation starts significantly earlier than sexual development, so intrinsic signals play an important role for the decision of the fungus to start cleistothecia formation. Accordingly, there has to be crosstalk between regulatory proteins specific for the asexual cycle and the sexual cycle of development. Some environmental parameters, which have to be perceived and translated into internal signals, are essential for both differentiation pathways. Numerous mutant strains altered in genes essential for both spore-producing developmental programs which have been described as defective in sexual sporulation, had originally been isolated for other characteristic phenotypes.

These genes include e.g. some of the *flu* (= fluffy) genes or their suppressors which influence the development of the vegetative mycelium as well as sporulation program (Wieser et al., 1997). Flu mutations generate colonies with profuse aerial hyphae, giving them the appearance of cotton wool (Wieser et al., 1994). Genetic analysis of *flu* genes and their suppressors revealed several elements of signalling pathways. Although initially described as defective in asexual sporulation, the *flu* phenotype is typically correlated with the inability to perform the sexual cycle, indicating that the gene products exert a connecting role between the two developmental programs.

In addition, elements of heterotrimeric G-proteins have been identified which are involved in development. The *sfaD* gene encodes the β–subunit (Rosen et al., 1999), *fadA* the α–subunit of a heterotrimeric G-protein (Adams et al., 1992). The *flbA* gene, a homologue of yeast SST2, encodes a RGS protein (= regulator of G-protein signalling) and seems to antagonize the action of the heterotrimeric G-protein (Yu et al., 1996). The major role of this G-protein might be to decide between growth as vegetative mycelium and the initiation of a developmental program like sporulation. It is unclear in *A. nidulans* whether the isolated heterotrimeric G-protein is connected to the cAMP-dependent PKA (protein kinase A) pathway. This connection exists in budding yeast between response to the nutritional situation in the environment and initiation of a development program, the filamentous pseudohyphal growth (Mösch & Fink, 1997; Kübler et al., 1997). The gene product of *flbE* is another protein which is presumably involved in signal transduction and required for developmental processes, but its exact molecular function is unknown.



Revised from Braus et al., 2002

Fig. 2.3 Determinants influencing fruiting body formation in *A. nidulans*. Environmental factors are shown in boxes, whereas relevant genetic determinants are presented in the inner boxes (see text for details). The electron micrograph shows a mature *A. nidulans* cleistothecium (**C**) surrounded by Hülle cells (**H**) was taken from Scherer & Fischer (1998).

The RasA, a small G-protein encoded by a homologue of the *ras* genes, has been shown to be essential for regulating an ordered developmental program. The active GTP-bound and the inactive GDP-bound form of the protein have been mimicked by the construction of dominant alleles with the appropriate mutations. The overexpression of constitutive inactive *rasA* alleles resulted in an acleistothecial phenotype (Hoffmann et al., 2000).

Several transcription factors have been identified which are involved in asexual or/and sexual development of *A. nidulans*. For the asexual pathway, the most prominent genes are *brlA* and *abaA*. *brlA* encodes a C2/H2 Zinc finger protein and overexpression of either *flbC* or *flbD* in submerged hyphae activates its expression (Adams, et al., 1998; Wieser & Adams, 1995). *abaA* encodes a protein with an ATTS/TEA domain which is conserved among other members of the family. The *abaA* gene product acts downstream of *brlA*. These two key regulatory proteins seem to be specific for conidiation without obvious influence on cleistothecia formation. In contrast to this, *medA* and *stuA* are two modifier genes of development where mutant alleles exist which exhibit clear effects on asexual as well as on sexual differentiation with the *medA* gene product

having a more general role influencing both sporulation pathways. The corresponding wild type protein is responsible for the correct temporal expression of both transcripts of the asexual regulator *brlA* and also functions as coactivator required for normal levels of *abaA* expression (Busby et al., 1996). Mutations in the *medA* result in aberrant conidiophores with branching chains of metulae, delayed conidiophores (Clutterbuck, 1969). The *medA* mutant strains produce only Hülle cells during the sexual cycle. The *stuA* mutant strains are completely acleistothecial and exhibit spatially abnormal conidiophores with spore production from the vesicles (Miller et al., 1991). Another transcription factor DopA is also involved in asexual and sexual sporulation. The deletion of *dopA* in *A. nidulans* results in several morphologically distinguishable defects: vegetative hyphae and conidiophores show an abnormal morphology, the sexual cycle is abolished, suggesting a very early block in sexual development (Pascon & Miller, 2000). Deletion of the transcription factor *nsdD* prevents fruiting body development and Hülle cell formation. In contrast, when the *nsdD* gene was overexpressed, sexual-specific organ (Hülle cells) was formed even in submerged culture, which normally completely blocked sexual development, and the number of cleistothecia was also dramatically increased on solid medium. These results lead to propose that the *nsdD* gene functions in activating sexual development of *A. nidulans*. *A. nidulans steA* encodes a protein with a homeodomain 63-75% identical to those of other Ste12 proteins, with greatest similarity to Ste12α of *F. neoformans*. SteA and Ste12α lack the pheromone induction domain found in budding yeast Ste12, but have C-terminal C2/H2-Zn+2 finger domains not present in the other Ste12 proteins. A *ΔsteA* strain is sterile and differentiates neither ascogenous tissue nor fruiting bodies (cleistothecia). However, the development of sexual cycle-specific Hülle cells is unaffected. Filamentous growth, conidiation and the differentiation of PH-like (pseudohaphae-like) asexual reproductive cells (metulae and phialides) are normal in the deletion strain. Northern analysis of key regulators of asexual and sexual reproduction cycles support the observation that although SteA function is restricted to the sexual cycle, cross regulation between the two developmental pathways exists (Vallim et al., 2000). RosA (repressor of sexual development) is a $Zn(II)_2Cys_6$ transcription factor characterized recently in our group. (Vienken, 2003). Overexpression of *rosA* in *A. nidulans* led to a reduction of the hyphal growth rate, a complete block of development and the proliferation of aerial mycelium giving the colonies a fluffy, cotton-like appearance. Deletion of the gene induced the production of masses of Hülle cells in submerged culture, which has never been observed in the wild type. Because of the observation that overexpression of the transcription factors NsdD or VeA induce Hülle cell

formation in liquid culture comparable to the deletion of *rosA*, it was proposed that a coordianated action of activators such as NsdD and VeA and the repressor RosA mediate the transition from vegetative growth and asexual development to sexual development.

The *A. nidulans* sexual cycle consists of several developmental programs which are interconnected: the formation of ascogenous hyphae, asci, and ascospores; the formation of Hülle cells; and the formation of the fruiting body envelope surrounding the asci. Since several *A. nidulans* mutant strains exhibit only Hülle cells, the formation of this specific cell type can be uncoupled from the other processes. Furthermore, sexual and asexual development seem to be interconnected as the two programs presumably share regulatory elements necessary for both sporulation programs. Accordingly, cleistothecia formation depends on the regulation of a large number of genes. In addition, the molecular analysis of a number of mutant alleles of genes which might play a role in cleistothecia formation will increase within the years.

2.3 Objective of this study

As described above, although a few genes involved in cleistothecium differentiation were identified, the process of sexual development is still poorly understood. In this study, the functions of three genes were mainly analysesd using a reverse genetic approach. Two of them, α-1,3-glucanase (mutanase, *mutA*) and a high-affinity hexose transporter (*hgtA*) which were discovered in a differential cDNA library (Scherer, 2001), were further analysed. In the search for factors of upstream signalling pathways triggering sexual development of *A. nidulans*, a MAP kinase kinase kinase (*steC*) was also studied.

2.3.1 Carbon cycle related to α-1,3-glucanase (mutanase) and high-affinity hexose transporters in sexual development of *A. nidulans*

The cell wall plays an important role in the growth and development of fungi. In addition to its function as the primary osmotic barrier of the cell, the temporal and spatial regulation of wall polymer synthesis is critical to the morphogenesis of the cell types characteristic of many fungi. During vegetative growth of *A. nidulans*, the mycelium stores large amounts of α-1,3-glucan (mutan), which occupies most of the alkali-soluble fraction (about 22% dry weight of complete cell wall) in the cell wall of *A. nidulans* (Zonneveld, 1971; 1972a). Later when the external

glucose supply is depleted, the glucan is broken down as glucose by an exo-splitting α-1,3-glucanase (mutanase) and reutilised as a carbon and energy source for cleistothecium formation (Zonneveld, 1972b; 1974)(Fig. 2.4). A mutant that lacks α-1,3-glucan is acleistothecial (Martinelli & Bainbridge, 1974; Polacheck & Rosenberger, 1977). It is assumed that the requirement of α-1,3-glucan for the formation of cell walls of the cleistothecium depends on increased α-1,3-glucanase activity, α-1,3-glucanase activity correlates with the density of cleitothecia. An *A. nidulans* mutant strain exhibiting an increased density of cleistothecia has been isolated which showed increased α-1,3-glucanase activity (Zonneveld, 1974). In this study, I characterized the α-1,3-glucanase gene (*mutA*) for α-1,3-glucan degradation, which is found in substractive hybridization library (SSH)(Scherer, 2001). It was shown that the gene is specifically expressed in Hülle cells, and is dispensable for sexual development.

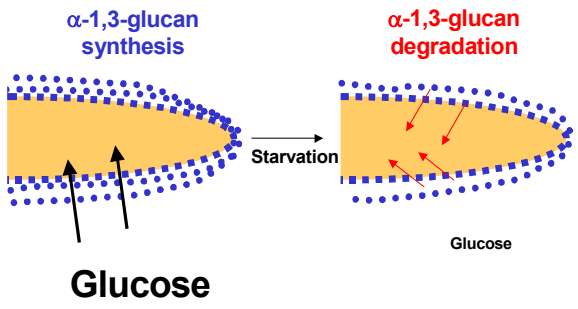


Fig. 2.4 Carbon cycle including the cell wall as storage compartment. *A. nidulans* synthesizes α-1,3-glucan (mutan) using the carbon source such as glucose during vegetative growth and deposits it as cell wall material. After depletion of external glucose, α-1,3-glucan can be degraded by α -1,3-glucanase and reutilised as a carbon and energy source during the formation of cleistothecia (Zonneveld, 1972b; 1974).

The initial step in glucose metabolism is the uptake of glucose, which is carried out by hexose transporter proteins localized in the plasma membrane (Bisson et al., 1993; Boles & Hollenberg, 1997; Kruckeberg, 1996; Ozcan & Johnston, 1999). Eukaryotic and bacterial sugar transporter proteins are related in protein sequence and cellular topology, forming the sugar permease superfamily (Bisson et al., 1993). In the yeast *Saccharomyces cerevisiae*, hexose transporter (HXT) proteins transport glucose across the plama membrane. The HXT proteins are encoded by a multigene family with 20 members, of which HXT1-4p and HXT6-7p are the major hexose transporters. The remaining HXT proteins have other or unknown function (Ozcan & Johnston, 1999; Reifenberger et al., 1995; Wieczorke et al., 1999). The existence of such a multigene

family of glucose transporters in this yeast resembles the situation in the mammalian systems (Gould & Bell, 1990; Weierstall et al., 1999), in which six glucose carriers have already been identified. In *A. nidulans,* it is anticiptated that the extracellularly monosaccharide released by the action of mutanase can be taken up by the cells and transferred to the metabolism by hexose transporters. Indeed in the subtractive hybridisation library (Scherer, 2001), in which also the mutanase gene was discovered, a fragment, whose translation sequence exhibited similarity to a high-affinity hexose transporter gene of the yeast, was found. This enable us to examine glucose utilization during sexual development was examined. Here a new *A. nidulans* gene, *hgtA* (high-affinity glucose transporter), which may code for the major high-affinity glucose carrier in this fungus was analysed.

2.3.2 MAP kinase cascade

Mitogen-activated protein kinases (MAP kinases) are ubiquitous among eukaryotes. MAP kinases are components of MAP kinase cascades, which are major signalling modules by which cells transduce extracellular cues into intracellular responses (Gustin et al., 1998; Stork & Schmitt, 2002). Originally they were described as protein kinases, which were transiently activated by a variety of mitogens, including insulin or growth factors and are thus implicated in cell proliferation and regulation of the cell cycle. Misregulation in animal cells leads to inappropriate activation of cell division and might result in the development of cancer (Schramek, 2002). The basic mechanism of signal transduction appears to be very similar in different MAP kinase cascades, namely a sequential activation of protein kinases upon the external stimulation with a signal. One early kinase after signal recognition, is a MAP kinase kinase kinase, which phosphorylates a MAP kinase kinase at two amino acid residues. The latter kinase in turn activates a MAP kinase, again by dual phosphorylation. The phosphorylated MAP kinase triggers the activity of transcription factors, and thereby the external signal is transmitted from the surface of the cell into the nucleus.

The evolutionary conservation of MAP kinase signalling pathways allows to use lower eukaryotes such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* as models to unravel the molecular and biochemical functions of the components (Herskowitz, 1995). In *S. cerevisiae* at least 6 different MAP kinase cascades exist which differ in the signals, which are perceived and transmitted, in the activation of the specific MAP kinase and ultimately a specific

transcription factor (Fig. 2.5)(Gustin, et al., 1998; Hohmann, 2002). The cascades are involved in mating, nutrient sensing and pseudohyphal growth, osmoregulation and stress adaptation, cell integrity, and ascospore formation. Thus the cellular responses are as diverse as the induction of mating upon pheromone perception and the synthesis of compatible solutes upon osmotic stress. Nevertheless, some signalling molecules are used in different cascades. Likewise the MAPKK kinase Ste11 is involved in mating, pseudohyphal growth and osmoregulation. The regulation of the specificity of each cascade and the prevention of cross talk between them is one important and largely unsolved question (Sabbagh et al., 2001). In plant or human pathogenic fungi MAP kinase cascades are involved in triggering the pathogenic program and the adaptation to the host-specific environmental conditions (Lengeler et al., 2000; Mayorga & Gold, 1999; Müller et al., 1999; Xu, 2000).

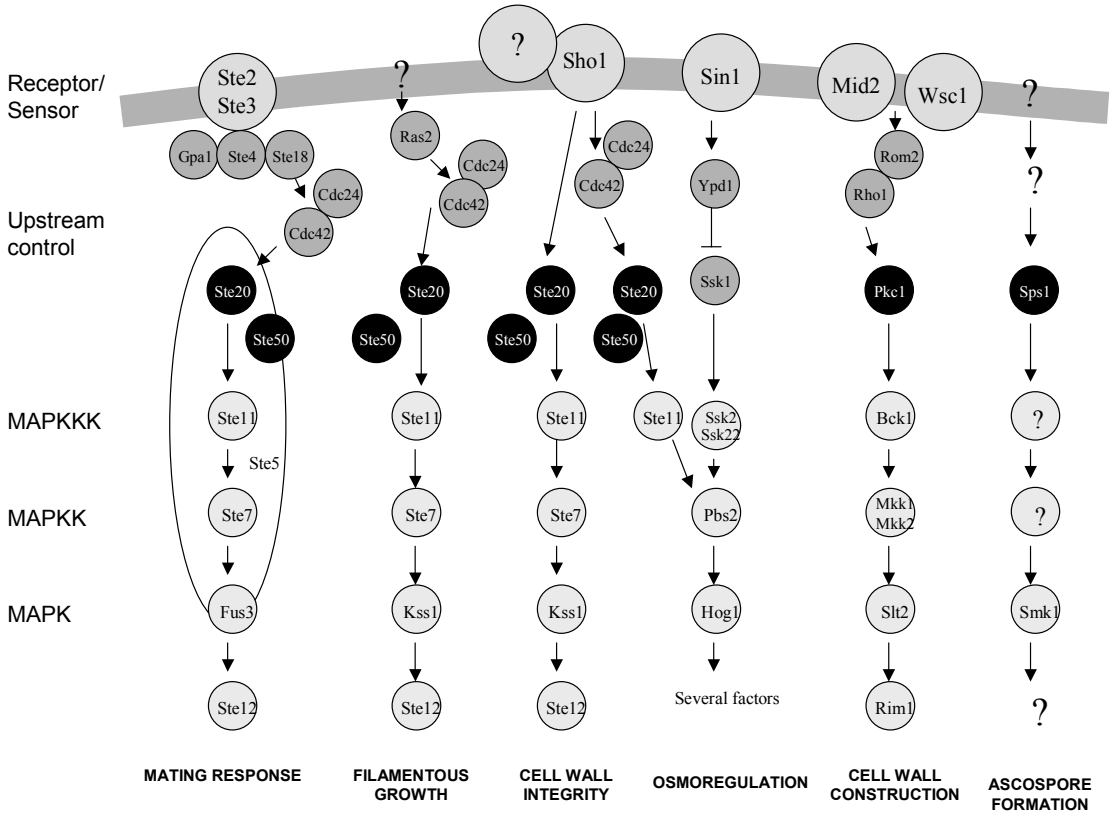


Fig. 2.5 Six *S. cerevisiae* MAP kinase pathways. The MAPKKK, MAPKK, and MAP kinase cascades regulating mating responses, filamentous growth, cell integrity, and osmoregulation in vegetative cells grown under different conditions. The *SMK1* pathway is expressed only during ascospore formation (Revised from Hohmann, 2002)

MAP kinase cascades are involved in major developmental transitions in the life cycle of the unicellular fungus *S. cerevisiae* (Fig. 2.5). In comparison to *S. cerevisiae,* the life style of the filamentous fungus *A. nidulans* is much more complex (Adams, et al., 1998; Braus, et al., 2002; Fischer, 2002). Unicellular spores initiate polarized growth and emerge germtubes at one pole of the spore. This hyphae extends by continuous elongation of the hyphal tip. The mycelium is metabolically very versatile and can adapt to many different environmental conditions, such as high osmolarity, different temperatures or desiccation. Besides hyphal growth *A. nidulans* can undergo different developmental programs. After about 20 h of vegetative growth, asexual reproductive structures, called conidiophores emerge into the air. From a vesicle on top of a stalk two layers of unicellular, uninucleate cells, the metulae and phialides are generated in a budding-like process. The phialides are the spore-producing cells, which continuously protrude conidiospores. The growth pattern in the conidiophore resembles the pseudohyphal growth type of *S. cerevisiae* (Gimeno et al., 1992). After about 5-6 days and after depletion of the carbon source, the mycelium of the fungus enters the sexual cycle. *A. nidulans* is a homothallic fungus and does not require a mating partner although a cross to other strains is favoured. The sexual developmental pathway leads to the formation of highly differentiated structures, the cleistothecia, in which karyogamy and subsequent meiosis occurs to generate haploid ascospores. The developmental decisions are partly triggered by a pheromone system consisting of interconvertible C-18 fatty acids (Champe & El-Zayat, 1989). However, the exact function of the pheromones as well as their perception and cellular responses are largely unknown. To get insights into the involvement of MAP kinase cascades during the life cycle of *A. nidulans* the MAPKK kinase SteC, a homologue of *S. cerevisiae* Ste11, was studied.

3 Materials

3.1 Equipment and Chemicals

Table 3.1 Main equipment used in this study

Equipment	Type	Manufacturer
Centrifuge	SORVALL RC 5B plus (HB-6)	SORVALL, Bad Homburg,
with rotors	SORVALL ultra pro80	
	SORVALL RC 28S	
	Centrifuge 5403	Eppendorf, Hamburg
Electroporation apparatus	Gene Pulser II, Pulse Controller	Bio-Rad, Munich
Electrotransfer apparatus	Mini Trans-blot Electrophoretic Transfer Cell	Bio-Rad, Munich
Hybridization oven	Personal Hyb™	Stratagene, Heidelberg
PCR machines	Rapid Cycler	Idaho Technology
	Personnal Cycler	Biometra, Goettingen
SDS-PAGE apparatus	Mini Protein II	Bio-Rad, Munich
UV-cross Linker	UV Stratalinker 2400	Stratagene, Heidelberg
UV/Visible spectrophotometer	Ultrospec 3100 *pro*	Amersham Pharmacia Biotech, Freiburg

Table 3.2 Kits used in this study

Kit	Manufacturer
BM Chemimminescence Blotting Substrate (POD)	Roche, Mannheim
DNeasy Plant Kit	Qiagen, Hilden
Nucleobond® AX	Macherey-Nagel, Düren
RNeasy Mini Kit	Qiagen, Hilden
QIAEX® II Gel Extraction Kit (150)	Qiagen, Hilden
QIAquick® PCR Purification Kit	Qiagen, Hilden

Chemicals were purchased from Boehringer (Mannheim), Merck (Darmstadt) Sigma (Diesenhofen), Roth (Karlsruhe), Biomol (Hamburg) and Difco Laboratories (Detroit, MI, USA). Restriction enzymes and other DNA-modifying enzymes from Amersham (Braunschweig), New England Biolabs (NEB) (Frankfurt) or Invitrogen (NV Leek, The Netherlands). The enzymes for PCR from Qiagen (Hilden) or Promaga (Mannheim). Radionucleotide [α-^{32}P]-dATP from Hartmann Analytics (Braunschweig). The autoradiographic film from Kodak (Rochester, NY, USA) or Fuji (New RX, Fuji, Japan). Filter (Miracloth) from Calbiochem-Novabiochem (Bad Soden / Ts.). Anti-phospho MAPK antibodies from New England Biolabs.

3.2 Media

Ingredients were added to ddH_2O, poured into bottles with loosen caps and autoclaved 20 min at 15 lb/in^2, For solid media, 1.5% (w/v) agar was added in media. Glassware and porcelain was sterilized for 3 h at 180°C. Heat-sensitive solutions such as antibiotics, amino acids and vitamins were filter-sterilized with 0.22 μm pore filter membranes (Millipore, France)

Standard media for *Escherichia coli* according to Sambrook & Russel (1999) were shown in Table 3.3, and supplements in Table 3.4.

Table 3.3 Media for *E. coli*

Medium	Ingredients (1 liter)
LB	10 g Bacto-Trypton; 5 g Bacto-Yeast Extract; 10 g NaCl
SOC	20 g Bacto-Trypton; 1 g Bacto-Yeast Extract; 5 g NaCl; 0.185 g KCl; 2.03 g $MgCl_2$ x $7H_2O$; 2.46 g $MgSO_4$ x $7H_2O$; 3.6 g Glucose

Table 3.4 Antibiotics and supplements for *E. coli* media

Substance	End concentration
Ampicillin (Ap)	100 μg/ml
Kanamycin (Km)	50 μg/ml
X-Gal	40 μg/ml
IPTG	8 μg/ml

Minimal and complete media for *A. nidulans* growth were prepared according to the protocols (Pontecorvo et al., 1953). For protoplast transformation of *A. nidulans,* 0.6 M KCl as osmoprotection substance was added into minimal media (Table 3.5). The supplemented vitamins, amino acids and ncleotides for auxotrophic *A. nidulans* strains were listed in Table 3.6.

Table 3.5 Media and stock solutions for *A. nidulans*

Media or Stock	Preparation (per liter)
20 x Salt stock solution	120 g $NaNO_3$; 10.4 g KCl; 10.4 g $MgSO_4$ x $7H_2O$; 30.4 g KH_2PO_4
1000 x Microelement stock solution	22 g $ZnSO_4$ x $7H_2O$; 11 g H_3BO_3; 5 g $MnCl_2$ x $4H_2O$; 5 g $FeSO_4$ x $7H_2O$; 1.6 g $CoCl_2$ x $5H_2O$; 1.6 g $CuSO_4$ x $5H_2O$; 1.1 g $(NH_4)_6Mo_7O_{24}$ x $4H_2O$; 50 g Na_4 EDTA; adjust to pH 6.5-6.8 using KOH
Minimal medium (MM)	50 ml Salt stock solution; 1 ml Microelement stock solution; 20 g Glucose; adjust to pH 6.5 using 10 N NaOH
Complete medium (CM)	Minimal medium with 2 g Peptone; 1 g Yeast extract; 1 g Casamino-acids; 1 ml Vitamin stock solution; 1 ml Microelement stock solution; adjust to pH 6.5 using 10 N NaOH

Table 3.6 Vitamins, amino acids and medium components

Component	Stock Concentration	Volume per liter
Biotin	0.05 %	1ml
PABA	0.1%	1ml
Pyridoxin-hydrochloride	0.1 %	1 ml
Arginine	500 mM	10 ml
Uracil	-	1 g
Uridine	500 mM	10 g

3.3 *A. nidulans* and *E. coli* strains

Table 3.7 *A. nidulans* and *E. coli* strains used in this study

Strain	Genotype	Source
FGSC26	*biA1; veA1*	FGSC, Kansas, USA
FGSCA4	wild type	FGSC, Kansas, USA

SRF200	*pyrG89; ΔargB::trpCΔB; pyroA4; veA1*	(Karos and Fischer, 1999)
GR5	*pyrG89; wA3; pyroA4; veA1*	G. May, Houston, TX, USA
RMSO11	*pabaA1, yA2; ΔargB::trpCΔB; veA1*	(Stringer et al., 1991)
AJC48	*biA1; medA26; veA1*	J. Clutterbuck, Glasgow, UK
SHW1 and 44	RMSO11 transformed with *mutA::argB* (pMut-argB); homologous integration	This study
SHW26 and 29	RMSO11 transformed with *mutA::argB* (pMut-argB); no replacement of mutA; considered wild type	This study
SHW44-13	*pabaA1, yA2; veA1; mutA::argB* SWH44 crossed to SRF200; *mutA* replacement selected	This study
SHW-p-sgfp17 and 19	RMSO11 transformed with *mutA(p)::sgfp* (pMut-p-sgfp) and pDC1	This study
SHW-gpd3, 4, 5	RMSO11 transformed with *gpd(p)::mutA* (pMut-gpd) and pDC1	This study
SWHH11	SRF200 transformed with *hgtA::argB* (pHHRarg11), homologous integration	This study
SWHgfp1, 7	SRF200 transformed with *hgtA::sgfp* (pHHgfp4)	This study
SWTB	SRF200 transformed with pDC1, wild type phenotype	(Schier, 2001)
SWH33 and 35	RMSO11 transformed with *steC::argB* (pHSAB3); homologous integration	This study
SWH51, 57	SRF200 transformed with *steC::argB* (pHSAB3); homologous integration	This study
SWHSR3	*ΔsteC* strain SWH51 retransformed with pHSKS2	This study
SWHSGP4, 6, 8	*ΔsteC* strain SWH51 retransformed with pHSKP3	This study
SWHSGR3, 5, 8	*ΔsteC* strain SWH51 retransformed with pHSKR4	This study
SWHSGSa3, 4	*ΔsteC* strain SWH51 retransformed with pHSKSa4	This study
SWHSGSt2, 4, 8	*ΔsteC* strain SWH51 retransformed with pHSKSt4	This study
SWHSgfp3, 21	RMSO11 transformed by pHSRB-gfp1 and pDC1,	This study

Escherichia coli		
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F'proABlacIQZ.M15::Tn10 (TetR)]*	Stratagene, Heidelberg
Top10F'	*F'[lacIQ, Tn10 (TetR)] mcrA .(mrr-hsdRMS-mcrBC), O80 lacZ .M15.lacX74, deoR, recA1, araD139.(ara-leu)7679, galU, galK, rpsL, (StrR) endA1, nupG*	Invitrogen, Leek, Netherlands
GM2159	*thr-1, araC14, leuB6, DE(gpt-proA)62, lacY1, tsx-33, glnV44(AS), galK2(Oc), LAM-, hisG4(Oc), rpsL31(strR), dam-13::Tn9, xylA5, mtl-1, recF143, argE3(Oc), thi-1*	*E. coli* Genetic Stock Center, New Haven, CT, USA

3.4 Plasmids and Cosmids

Table 3.8 Plasmids and cosmids used in this study

Cosmids/ Plasmids	**Construction**	**Source**
pUC18	Cloning vector	MBI Fermentas, St. Leon-Rot
pBluescript KS$^-$	Cloning vector	Invitrogen (NV Leek, The Netherlands)
pCR®-Blunt	Cloning vector	Invitrogen (NV Leek, The Netherlands)
pCR2.1TOPO	Cloning vector	Invitrogen (NV Leek, The Netherlands)
pDC1	*A. nidulans argB* gene in pIC20R	(Aramayo *et al*., 1989)
pRG1	*N. crassa pyr-4* gene as selection marker	(Waring et al., 1989)
pHG2	mutanase containing cosmid	This study
pHGE5	7.3 kb *Eco*RV mutanase-containing fragment cloned into pCR®-Blunt	This study
pHGE5-Sac	pHGE5 digested with *Sac*I and religated	This study
pHW-arg	*argB* released with *Kpn*I-*Xho*I from pDC1 and cloned into corresponding sites in pBluescript	This study

pMut-arg	*argB* released with *Bam*HI from pHW-arg and cloned into *Bam*HI digested pHGE5-Sac	This study
pMS-gpd	*gpd* promoter inserted into *Bam*HI and *argB* into *Not*I of pBluescript KS$^-$	(Scherer, 2001)
pMut-gpd	*mutA* open reading frame cloned behind the *gpd* promoter	This study
pRF917	*alcA(p)*::*kinA*::*sgfp*, *sgfp* cloned as a *Not*I fragment	(Requena et al., 2001)
pMut-p-sgfp	1.8 kb *mutA* promoter fused with *sgfp* from pRF917 into *Eco*RV and *Not*I of pBluescript KS$^-$	This study
pH38E7	a *hgtA*-containing cosmid from *A.nidulans* PUI cosmid library	This study
pHHH1	4 kb *Hind*III *hgtA*-containing fragment was subcloned into PCR2.1TOPO	This study
pHHPS4	3 kb *Pst*I-*Sal*I fragment containing the promoter and partial N-terminal was subcloned into pBluescript KS$^-$	This study
pHHRarg11	*hgtA* deletion construct (stratagy see result 5.1.5)	This study
pHHgfp4	KS-Rev/Hex-Not1 amplyfied fagement (2.5 kb) from pHHPS4 were inserted into *Eco*RV of pBluescript KS$^-$, *sgfp* from pMut-p-sgfp was inserted into *Not*I	This study
pAG1	*steC*-containing cosmid	(Geißenhöner et al., 2001)
pHSKS2	8 kb *Kpn*I-*Sal*I *steC*-containing fragment from pAG1 cloned into pCR2.1TOPO	This study
pHSAB3	*Bam*HI-released *argB* from pHW-arg inserted into pHSKS2, *steC* deletion construct	This study
pHSRB-gfp1	4 kb *Eco*RV-*Bam*HI fragment containing *steC* promoter and N-terminal from pHSKS2 inserted into *Eco*RV-*Bam*HI and *sgfp* into *Not*I of pBluescript KS$^-$	This study
pHSKGP3	*gpd*::*steC* / *Pvu*II-*Kpn*I fragment in pMS-gpd	This study
pHSKGR2	*gpd*::*steC* / *Eco*RI-*Kpn*I fragment in pMS-gpd	This study

pHSKGSa4	*gpd*::s*teC* / *Sac*I-*Kpn*I fragment in pMS-gpd	This study
pHSKGSt4	*gpd*::s*teC* / *Stu*I-*Kpn*I fragment in pMS-gpd	This study

3.5 Oligonucleotides

All primers used in this study were synthesized by MWG Biotech (Ebersberg)

Table 3.9 Primers used for PCR

Glucan1	5'-CAGTAGACCAGCTGGTCAG-3'
Glucan2	5'-GGAGGTTCAGGAATAATCTC-3'
Glucan-ex1	5'-CGCATCCCAGGCAACACA-3'
Glucan-ex2	5'-CTGCACGCACCCTCCCTA-3'
glu-P1	5'-CTCGTCGTGGCTGTGTGGAT-3'
glu-P2	5'-GCGGCCGCCATTGCGGCGTCAGTTGCT-3'
glu-P3	5'-GCGTAAGGGTATGAGATGGT-3'
glu-P4	5'-AGCCACGCCGTATGAGGAAT-3'
glu-P5	5'-TTCGCGCGATACCCATCAGT-3'
glu-P6	5'-GCGCAAGGTCTTAACATTGCCT-3'
glu-P7	5'-TTCGAGATTTTCCAGCGAGCATC-3'
glu-P8	5'-GTTAAATAGATGCCCGTGTCGCT -3'
glu-P9	5'-CCAGTCAGTATCTCTCAAAGCCT-3'
KS-gfp	5'-CGACTCACTATAGGGCGAATT-3'
GluP1B	Biotin-5'-CTCGTCGTGGCTGTGTGGAT-3'
GluP6F	5'-CAATGTTAAGACCTTGCGCT-3'
GluP4B	Biotin-5'-AGCCACGCCGTATGAGGAAT-3'
GluP4F	5'-ACCTGCCACCGGAAGACACT-3'
HexA5’	5’-GTTTCGACATCTCGTCGATG-3’
HexA3’	5’-CCAGCGGTCCTTGCTCG-3’
HexB5’	5’-CAATTGTCGGGAATGAACGTC-3’
HexB3’	5’-GTTCAAGGCGGCGACAGTG-3’
HexR1	5'-CCTTGGTGTTTCTCTTCTTCCTG-3'
HexR2	5'-TGAAGATGGCTTTCAAGAAGTCCT-3'
HexR3	5'-CATGACCCTTGGTATGAAACCGT-3'

HexF	5'-GACCAGCAAGGGGATACCGT-3'
Hex-Not1	5'-CGCGGCCGCCATCTTCAAAGGCGGTGCT-3'
KS-Rev1	5'-GCCAAGCGCGCAATTAACCCTCACT-3'
Hex-ex2	5'-AATGGACTCGAGATTACACCGCCTTCTCCGGT-3'
SteC-R1	5'-ATGTCCCGTTCCCTTAGATTCA -3'
SteC-R8	5'-GTTCTGCGACATGGCTAAATGATCGTGTCC -3'
SteC-R9	5'-TCCTCTACGAATCCGGATCAATATCTCTCG -3'
SteC-F4	5'-TCCTTTCGCCCTGAATCCAT-3'
SteC-F6	5'-CTGGTCGCATTCGATAAGGT-3'
Ste11A	5'-CGAGAGCCAACAGGCGC-3'
Ste11B	5'-TCATCGGCGGTAGTTCC-3'
Ste11C	5'-GTCTTGATGCTGTGAGGTG-3'
Ste11D	5'-GGGTCAGGGTCCAAGCC-3'
Ste11-ex3	5'-CGTCCTCATCGTTACGTTGCT-3'
Ste11-ex2	5'-TTGGTTGTTTTGTCGCGTGAGT-3'

4 Methods

4.1 Growth conditions and storage of transformed *E. coli* and *A. nidulans* strains

Transformed *E. coli* was overnight cultivated on LB plates with appropriate antibiotics at 37°C. Liquid culture was inoculated from a single colony and incubated in LB medium containing appropriate antibiotics at 37°C with 250 rpm overnight shaking. Freshly grown bacterial suspension was adjusted to 15% end concentration of sterile glycerol and stored at –80°C.

The *A. nidulans* strains were grown on minimal or complete medium plates. Pieces of a colony were cut from an agar plate and suspended in 15-20% sterile glycerol and stored at –80˚C.

4.2 Transformation of *A. nidulans*

Standard procedures of *Aspergillius* protoplast transformation were used (Yelton et al., 1984).

4.2.1 Preparation of protoplast

A 500 ml volume of spore culture in minimal medium with appropriate components was shaked at 30ºC in water bath for 12-16 h until spores germinated. The culture was filtered through sterile miracloth followed by washing using Wash solution. The washed mycelium was collected into a sterile flask that was set on ice. Then 5 ml of Osmotic medium, 200 mg of GlucanX (Novozyme) in 1 ml sterile water and 6 mg BSA in 0.5 ml water was successively added into the flask. The digestion mixture in the flask was incubated at 30ºC in water bath for 1-3 h until enough protoplasts became free. The digestion mixture was transferred into a 30 ml corex tube and 10 ml of Trapping buffer was slowly added on top of the mixture, followed by a centrifugation at 5000 rpm for 15 min using a HB-6 rotor. Then a protoplast band was transferred into a new sterile tube, followed by washing two times using STC with centrifugation at 7000 rpm for 8 min. The protoplast pellet was gently resuspended in 200-1000 µl STC for transformation.

4.2.2 Protoplast transformation

100 µl of protoplasts in STC and 100 µl DNA (10 µg DNA filled with 100 µl STC) were mixed and incubated 25 min at room temperature in a falcon tube. Then, 2 ml PEG was added and the tube was rolled until the mixture was homogeneous, followed by 20 min incubation in room temperature. Finally, 8 ml STC was added and the entire mixture spread onto osmatically stabilized medium (MM + 0.6 M KCl) with appropriate selection markers. The plates were incubated at 37ºC until clear colonies were formed after 3-4 days.

Mycelium wash solution	0.6 M $MgSO_4$
Osmotic medium	1.2 M $MgSO_4$; 10 mM Na_3PO_4 buffer (pH 5.8)
Trapping buffer	0.6 M Sorbitol; 0.1 M Tris-HCl (pH7.0)
STC	1.2 M Sorbitol; 10 mM $CaCl_2$; 10 mM Tris-HCl (pH 7.5)
PEG	60% PEG 4000; 10 mM $CaCl_2$; 10 mM Tris-HCl (pH 7.5)

4.3 DNA and RNA manipulations

4.3.1 Plasmid DNA preparation from *E. coli* cells

An alkali-lysis method was used for the isolation of plasmid or cosmid DNA (Sambrook & Russel, 1999). For a small volume of liquid culture (Miniprep), 1.5 ml overnight culture was centrifuged 1 min at 13000 rpm, and the pellet resuspended in 200 µl Tris-EDTA buffer. Then 200 µl Alkali-lysis buffer was added, gently mixed, with the cell suspension, followed by the addition of 200 µl Neutralization buffer. After a 10 min centrifugation, plasmid DNA-containing supernatant was precipitated with 0.7 vol. isopropanol followed by 70% EtOH washing. After drying, the pellet was resuspended in TE buffer. For a large volume of liquid culture (50-100 ml; Midipreps), plasmid DNA was prepared using the Qiagen Midi Plasmid Purification Kit.

Plasmid DNA concentration was determined via absorption measurement with 260 and 280 nm in a spectrophotometer (Pharmacia LKB-UltrospecIII) in a quartz cuvette or compared the intensity of Ethidium bromide stained DNA bands on an agarose gel with the intensity of defined standards.

Tris-EDTA buffer	5 ml 1 M Tris-HCl (pH7.5); 2 ml 0.5 M EDTA (pH8.0); 10 mg RNAse in 100 ml
Alkali-lysis buffer	0.2 M NaOH; 1% SDS
Neutralization buffer	1.5 M K-Acetate (pH4.8)
TE buffer	10 mM Tris-HCl; 1 mM EDTA; pH8.0

4.3.2 Genomic DNA preparation from *A. nidulans*

For the preparation of *A. nidulans* genomic DNA, around 25 ml fresh liquid minimal media in a 9 cm plastic plate was inoculated using a spore suspension prepared the colony grown on an agar plate. The culture was incubated for 12-15 h at 37°C. Then, the mycelium was taken off with a spatula and pressed briefly until dry between paper towels, and put into liquid N_2. The frozen mycelium was grinded in liquid N_2 or kept at –80˚C until isolation. *A. nidulans* genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden). Finally, 5 µl DNA extraction was used to check quality and yield via running a 1% agarose gel.

4.3.3 Precipitation of DNA

Contamination by small nucleic acid fragments, protein and salt can be reduced to acceptable levels by precipitating the DNA. 2.5 volume of ethanol and 1/10 3.0 M NaAc (pH 5.2) were added into the DNA solution. The sample was mixed, kept at –80ºC for more than 10 min and centrifuged for 10 min at 10000 rpm. The supernatant was discarded and the pellet was washed with 70% EtOH, followed by centrifugation at 10000 rpm for 5-10 min. The purified DNA pellet was completely dried in a speed vacuum or at 50ºC for 10-20 min, and then dissolved in sterile water or TE buffer.

4.3.4 DNA electrophoresis through agarose gel

DNA electrophoresis through agarose gel is a standard method to separate, identify and purify DNA fragments. An agarose gel of 0.8-1.2% was prepared by boiling agarose in 0.5 or 1 x TAE buffer and pouring it into gel-making models. DNA samples were mixed with 1/10 10 x DNA loading buffer. DNA samples together with a DNA marker (*Eco*130I-cut λ DNA, MBI

Fermentas, St. Leon-Rot) were separated at 10 V/cm for 30 min-4 h depending the length of the gel in 0.5 or 1 x TAE buffer. Then, the gel was stained in 0.5 x TAE buffer with Ethidium bromide for 15-30 min. The DNA bands in the gel were visualizd by 302 nm UV light and pictures were taken with a camera (INTAS, Goettingen) connected to a video printer.

50 x TAE buffer (pH 8.0)	40 mM Tris-Acetate; 1 mM EDTA; pH 8.0
10 x Loading buffer	20% Ficoll 400; 0.1 M Na_2EDTA (pH 8.0); 1% SDS; 0.25% Bromphenol blue; 0.25% Xylene cyanol

4.3.5 Digestion of DNA by restriction endonucleases

DNA samples (200 ng – 1 µg) were digested by restriction endonucleases using corresponding reaction buffers. Enzyme, DNA, buffer and reaction time varied depending on the specific requirements (generally, 37˚C from 1 h to overnight). *A. nidulans* genomic DNA was generally digested for more than 2 h. For cloning, it was enough for 1-2 h. In the case where it was necessary to treat the same DNA sample with different enzymes, the digestion was carried out first in the buffer with low salt concentration or the buffer compatible to different enzymes.

4.3.6 PCR

Polymerase chain reaction (PCR) was accomplished with Taq (Gibco or Invitrogen), Expand (Boehringer) or Pfu (Promega) according to manufacturer protocols. The synthesis of oligo nucleotides was made by MWG Biotech (Ebersberg). As beginning oligonucleotide concentrations, 5-20 µM were used in a reaction volume of 10-100 µl. The PCR reaction took place in a Personal Cycler (Biometra), or a capillary Rapid Cycler (Idaho Technology, Idaho Falls, ID, USA). RT-PCR was carried out using SUPERSCRIPT^{TM} II RNase H$^-$ Reverse Transcriptase (Invitrogen) according to the manufacturer protocol.

A Standard PCR reaction in Rapid Cycler		A Standard PCR reaction in Personal Cycler	
1 µl	2.5 mM dNTP	5 µl	2.5 mM dNTP
1 µl	templete DNA	5 µl	templete DNA
1 µl	10 x buffer	5 µl	10 x buffer
1 µl	50 mM $MgCl_2$	5 µl	50 mM $MgCl_2$
1 µl	10 x BSA	5 µl each	5 µM Primer A and B
1 µl	10 x Ficcol	1 µl	Taq DNA polymerase
1 µl each	5 µM Primer A and B	24 µl	Autocloved ddH_2O
0.2 µl	Taq DNA polymerase		
1.8 µl	Autocloved ddH_2O		

4.3.7 DNA isolation from agarose gel

For DNA fragment isolation, 0.8% - 1% "low melting" gel was often used. The low melting gel separated by gel electrophoresis was stained in 0.5 x TAE with ethidium bromide. The appropriate DNA bands were cut out under UV light. The DNA purification was carried out according to the protocol of Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA). Alternatively, the DNA in normal agarose gels was isolated with the QIAEX II Gel Extraction System (Qiagen, Hilden).

4.3.8 Dephosphorylation of digested DNA

After the digestion with restriction enzymes, the vector was dephosphorylated by Shrimp alkaline phosphatase (SAP) to remove the phosphate group at the 5'-end which prevent religation of the vector. 0.1 unit / µM 5'-end with buffer was added into one sample. The mix was incubated around 30 min at 37ºC. Less SAP and shorter incubation time were used for the protruding 5' termini than for recessed 5' termini. If two enzymes with incompatible termini were used, the dephosphorylation process was omitted.

4.3.9 DNA ligation

DNA ligation was carried out using T4 ligase (Amersham-Pharmacia Biotech, Freiburg) at 16ºC or Fast Link™ System (Biozym, Hessisch Oldendorf) in a volume of 10-20 µl. The concentration of vector and insert DNA was measured on the basis of DNA marker. Around 50 ng vector was used in one ligation. The ratio of vector to insert was 1: 2-3 and 1:5-10 respectively for sticky and blunt end ligation. For the cloning of PCR products, it was often done to add restriction enzyme sites in both primers. In addition, PCR fragments were cloned blunt end into *Eco*RV or *Sma*I of pBluescript or into PCR-Blunt (Invitrogen, NV Leek, The Netherlands). For TA cloning, the PCR products amplified by *Taq* or *Taq*-containing polymerases were cloned into PCR2.1TOPO (Invitrogen).

4.3.10 DNA sequencing

DNA sequencing was done by commercial sequencing (MWG Biotech, Ebersberg).

4.3.11 Transformation of *E. coli*

The transformation of electrocompetent *E. coli* cells was done as described (Ausubel et al., 1995). A fresh single *E. coli* colony was cultured overnight in 37ºC. The culture was centrifuged, followed by repeatly washing using cool sterile water at 2ºC. The cells were resuspended in 10% glycerol and aliquot frozen at –80ºC for use. After desalting of ligation reaction solution, 2 µl ligation solution and 50 µl *E. coli* cells were mixed on ice and filled into transformation cuvette (PEQLAB, Erlangen). The plasmids were transformed by electroporation (Gene-Pulser, Bio-Rad) into electrocompetent *E. coli* cells XL1-Blue (Stratagene, La Jolla, USA). Alternatively, electrocompetent *E. coli* strain TOP10F' (Invitrogen, Leek, Nethelands) was used.

4.3.12 DNA-DNA hybridization (Southern blot analysis)

DNA-DNA hybridization (Southern blot analysis) according to Sambrook & Russel (1999) was accomplished using radioactive α-^{32}P-dATP or α-^{32}P-dCTP. The production of probes was made by means of random priming (usb, Freiburg) or in a PCR reaction with specific primers.

The DNA samples isolated in agarose gel were capillary transferred to the positively charged nylon filter (Biodyne A, Pall, Ann Arbor, MI, USA). The filter was cross-linked under UV radiation with a dose of 1.2 x 105 μJ (UV Stratalinker 2400, Stratagene, Heidelberg). The probe was purified through prespin Mobispin S-300 Column (Mo Bi Tec GmbH, Goettingen). The filter was prehybridized in Hybridization solution supplemented with 100 μg/ml Salmon sperm DNA more than 1 h at 68ºC and afterwards hybridized overnight with the probe at 68ºC, followed by stringent washing at 68ºC, first, 1 time in 2 x SSC / 0.1% SDS for 10 min, and then 2 times for each 10 min in 0.1-0.2 x SSC / 0.1% SDS. The detection was carried out by means of autoradiography using the films from Kodak (Rochester, NY, USA) or Fuji (New RX, Fuji, Japan). If the filter was reused, a process of stripping was carried out in 0.5% SDS at 95ºC for 2-4 times. The stripping result was radioactively checked.

Hybridization solution	5 x SSC; 1% skim milk; 0.1% Lauroylsarcocine sodium salt; 0.02% SDS
Acidic solution	0.25 M HCl
Denaturation solution	100 g NaOH; 438.3 g NaCl in 5 liter
Netralization solution	242 g Tris; 347 g NaCl in 4 l; pH 7.2
20 x SSC	441.3 g Na_3Citrate; 876.3 g NaCl in 5 l; pH 7.0
2 x Wash	100 ml 20 x SSC; 10 ml 10% SDS in 1 liter
0.2 x Wash	10 ml 20 x SSC; 10 ml 10% SDS in 1 liter

4.3.13 Isolation of total RNA from *A. nidulans*

For isolation of RNA in development stages, approximately 10^3 spores per 9 cm plate was inoculated onto complete medium plates covered with sterile preserving membrane (Ostmann, Bielefeld). Alternatively, a 500 ml CM liquid culture inoculated by spore suspension from one plate was shaken at 200 rpm for 14 h at 37ºC, 50 ml of liquid culture was vacuum filtered through miracloth, the filtered mycelium was put on CM agar plates, incubated at 37ºC. Then, the mycelium together with membrane or miracloth was harvested at defined times, removed extra water between paper towels, frozen in liquid nitrogen and grinded in a mortar. RNA isolation from grinded mycelium powder was carried out with TRIZOL (Gibco or Invitrogen) according to manufacturer protocol. The RNA was finally dissolved in 40-50 μl sterile DEPC H_2O with 0.5 U/μl RNase inhibitor (Promega, Mannheim). The RNA concentration was measured in a

spectrophotometer (Pharmacia LKB, UltrospecIII). The RNA samples were diluted to 2 μg/μl with DEPC H_2O containing RNase inhibitor and kept at –80°C.

4.3.14 DNA-RNA hybridization (Northern blot analysis)

DNA-RNA hybridization (Northern blot) was accomplished as described (Sambrook & Russel, 1999). The RNA was denatured with formamide and separated in denaturing formaldehyde agarose gel, followed by capillary transfer to a positively charged nylon membrane (Biodyne Plus, Pall, Ann Arbor, MI, USA). For size estimation, RNA marker of Promega company (Mannheim) was used. The filter was cross-linked as for Southern blots. Then, the membrane was stained by Methylene blue and washed with H_2O. The two clear rRNA bands should appear. The picture was taken via a camera (INTAS, Goettingen) and video printer (Ann Arbor, MI, USA). The filter was destained in Destaining solution. The filter was prehybridized in Northern hybridization solution with 100 μg/ml Salmon sperm DNA more than 1 h at 42°C and afterwards hybridized overnight with the probe at 42°C, followed by stringent washing at 65-68°C, 1 time in 2 x SSC / 0.1% SDS for 10 min, and then 2 times for each 10 min in 0.5 x SSC / 0.1% SDS. The detection was carried out as in Southern blots.

DEPC water	0.1% DEPC, stir overnight, autoclave
10 x MOPS	0.4 M MOPS (pH7.0); 0.1 M Sodium acetate; 0.01 M EDTA; autoclave
RNA sample buffer	100 μl Formamide; 38 μl 37% Formaldehyde; 20 μl 10 x MOPS; 42 μl DEPC water; 20 μl RNA loading buffer
RNA loading buffer	80% formamide; 1 mM EDTA; 0.1% Bromphenol blue; 0.1% xylene cyanol
Northern staining solution	0.03% Methylene blue in 0.3 M Na-Acetate
Northern destaining solution	1% SDS; 1 x SSC
100 x Denhardt's solution	10 g Ficoll 400; 10 g polyvinylpyrrolidone; 10 g BSA (Pentax fraction V); H_2O to 500 ml, filter and store at –20°C in 25 ml aliquots
Prehybridization / Hybridization solution	5 x SSC; 1% SDS; 5 x Denhardts; 50% formamide

4 Methods

4.1 Growth conditions and storage of transformed *E. coli* and *A. nidulans* strains

Transformed *E. coli* was overnight cultivated on LB plates with appropriate antibiotics at 37°C. Liquid culture was inoculated from a single colony and incubated in LB medium containing appropriate antibiotics at 37°C with 250 rpm overnight shaking. Freshly grown bacterial suspension was adjusted to 15% end concentration of sterile glycerol and stored at –80°C.

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Standard procedures of *Aspergillius* protoplast transformation were used (Yelton et al., 1984).

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A 500 ml volume of spore culture in minimal medium with appropriate components was shaked at 30ºC in water bath for 12-16 h until spores germinated. The culture was filtered through sterile miracloth followed by washing using Wash solution. The washed mycelium was collected into a sterile flask that was set on ice. Then 5 ml of Osmotic medium, 200 mg of GlucanX (Novozyme) in 1 ml sterile water and 6 mg BSA in 0.5 ml water was successively added into the flask. The digestion mixture in the flask was incubated at 30ºC in water bath for 1-3 h until enough protoplasts became free. The digestion mixture was transferred into a 30 ml corex tube and 10 ml of Trapping buffer was slowly added on top of the mixture, followed by a centrifugation at 5000 rpm for 15 min using a HB-6 rotor. Then a protoplast band was transferred into a new sterile tube, followed by washing two times using STC with centrifugation at 7000 rpm for 8 min. The protoplast pellet was gently resuspended in 200-1000 µl STC for transformation.

4.2.2 Protoplast transformation

100 µl of protoplasts in STC and 100 µl DNA (10 µg DNA filled with 100 µl STC) were mixed and incubated 25 min at room temperature in a falcon tube. Then, 2 ml PEG was added and the tube was rolled until the mixture was homogeneous, followed by 20 min incubation in room temperature. Finally, 8 ml STC was added and the entire mixture spread onto osmatically stabilized medium (MM + 0.6 M KCl) with appropriate selection markers. The plates were incubated at 37ºC until clear colonies were formed after 3-4 days.

Mycelium wash solution	0.6 M $MgSO_4$
Osmotic medium	1.2 M $MgSO_4$; 10 mM Na_3PO_4 buffer (pH 5.8)
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4.3 DNA and RNA manipulations

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Plasmid DNA concentration was determined via absorption measurement with 260 and 280 nm in a spectrophotometer (Pharmacia LKB-UltrospecIII) in a quartz cuvette or compared the intensity of Ethidium bromide stained DNA bands on an agarose gel with the intensity of defined standards.

Tris-EDTA buffer	5 ml 1 M Tris-HCl (pH7.5); 2 ml 0.5 M EDTA (pH8.0); 10 mg RNAse in 100 ml
Alkali-lysis buffer	0.2 M NaOH; 1% SDS
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TE buffer	10 mM Tris-HCl; 1 mM EDTA; pH8.0

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For the preparation of *A. nidulans* genomic DNA, around 25 ml fresh liquid minimal media in a 9 cm plastic plate was inoculated using a spore suspension prepared the colony grown on an agar plate. The culture was incubated for 12-15 h at 37°C. Then, the mycelium was taken off with a spatula and pressed briefly until dry between paper towels, and put into liquid N_2. The frozen mycelium was grinded in liquid N_2 or kept at –80˚C until isolation. *A. nidulans* genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden). Finally, 5 µl DNA extraction was used to check quality and yield via running a 1% agarose gel.

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4.3.4 DNA electrophoresis through agarose gel

DNA electrophoresis through agarose gel is a standard method to separate, identify and purify DNA fragments. An agarose gel of 0.8-1.2% was prepared by boiling agarose in 0.5 or 1 x TAE buffer and pouring it into gel-making models. DNA samples were mixed with 1/10 10 x DNA loading buffer. DNA samples together with a DNA marker (*Eco*130I-cut λ DNA, MBI

Fermentas, St. Leon-Rot) were separated at 10 V/cm for 30 min-4 h depending the length of the gel in 0.5 or 1 x TAE buffer. Then, the gel was stained in 0.5 x TAE buffer with Ethidium bromide for 15-30 min. The DNA bands in the gel were visualizd by 302 nm UV light and pictures were taken with a camera (INTAS, Goettingen) connected to a video printer.

50 x TAE buffer (pH 8.0)	40 mM Tris-Acetate; 1 mM EDTA; pH 8.0
10 x Loading buffer	20% Ficoll 400; 0.1 M Na_2EDTA (pH 8.0); 1% SDS; 0.25% Bromphenol blue; 0.25% Xylene cyanol

4.3.5 Digestion of DNA by restriction endonucleases

DNA samples (200 ng – 1 µg) were digested by restriction endonucleases using corresponding reaction buffers. Enzyme, DNA, buffer and reaction time varied depending on the specific requirements (generally, 37˚C from 1 h to overnight). *A. nidulans* genomic DNA was generally digested for more than 2 h. For cloning, it was enough for 1-2 h. In the case where it was necessary to treat the same DNA sample with different enzymes, the digestion was carried out first in the buffer with low salt concentration or the buffer compatible to different enzymes.

4.3.6 PCR

Polymerase chain reaction (PCR) was accomplished with Taq (Gibco or Invitrogen), Expand (Boehringer) or Pfu (Promega) according to manufacturer protocols. The synthesis of oligo nucleotides was made by MWG Biotech (Ebersberg). As beginning oligonucleotide concentrations, 5-20 µM were used in a reaction volume of 10-100 µl. The PCR reaction took place in a Personal Cycler (Biometra), or a capillary Rapid Cycler (Idaho Technology, Idaho Falls, ID, USA). RT-PCR was carried out using SUPERSCRIPT^{TM} II RNase H$^-$ Reverse Transcriptase (Invitrogen) according to the manufacturer protocol.

A Standard PCR reaction in Rapid Cycler		A Standard PCR reaction in Personal Cycler	
1 µl	2.5 mM dNTP	5 µl	2.5 mM dNTP
1 µl	templete DNA	5 µl	templete DNA
1 µl	10 x buffer	5 µl	10 x buffer
1 µl	50 mM $MgCl_2$	5 µl	50 mM $MgCl_2$
1 µl	10 x BSA	5 µl each	5 µM Primer A and B
1 µl	10 x Ficcol	1 µl	Taq DNA polymerase
1 µl each	5 µM Primer A and B	24 µl	Autocloved ddH_2O
0.2 µl	Taq DNA polymerase		
1.8 µl	Autocloved ddH_2O		

4.3.7 DNA isolation from agarose gel

For DNA fragment isolation, 0.8% - 1% "low melting" gel was often used. The low melting gel separated by gel electrophoresis was stained in 0.5 x TAE with ethidium bromide. The appropriate DNA bands were cut out under UV light. The DNA purification was carried out according to the protocol of Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA). Alternatively, the DNA in normal agarose gels was isolated with the QIAEX II Gel Extraction System (Qiagen, Hilden).

4.3.8 Dephosphorylation of digested DNA

After the digestion with restriction enzymes, the vector was dephosphorylated by Shrimp alkaline phosphatase (SAP) to remove the phosphate group at the 5'-end which prevent religation of the vector. 0.1 unit / µM 5'-end with buffer was added into one sample. The mix was incubated around 30 min at 37ºC. Less SAP and shorter incubation time were used for the protruding 5' termini than for recessed 5' termini. If two enzymes with incompatible termini were used, the dephosphorylation process was omitted.

4.3.9 DNA ligation

DNA ligation was carried out using T4 ligase (Amersham-Pharmacia Biotech, Freiburg) at 16ºC or Fast Link™ System (Biozym, Hessisch Oldendorf) in a volume of 10-20 µl. The concentration of vector and insert DNA was measured on the basis of DNA marker. Around 50 ng vector was used in one ligation. The ratio of vector to insert was 1: 2-3 and 1:5-10 respectively for sticky and blunt end ligation. For the cloning of PCR products, it was often done to add restriction enzyme sites in both primers. In addition, PCR fragments were cloned blunt end into *Eco*RV or *Sma*I of pBluescript or into PCR-Blunt (Invitrogen, NV Leek, The Netherlands). For TA cloning, the PCR products amplified by *Taq* or *Taq*-containing polymerases were cloned into PCR2.1TOPO (Invitrogen).

4.3.10 DNA sequencing

DNA sequencing was done by commercial sequencing (MWG Biotech, Ebersberg).

4.3.11 Transformation of *E. coli*

The transformation of electrocompetent *E. coli* cells was done as described (Ausubel et al., 1995). A fresh single *E. coli* colony was cultured overnight in 37ºC. The culture was centrifuged, followed by repeatly washing using cool sterile water at 2ºC. The cells were resuspended in 10% glycerol and aliquot frozen at –80ºC for use. After desalting of ligation reaction solution, 2 µl ligation solution and 50 µl *E. coli* cells were mixed on ice and filled into transformation cuvette (PEQLAB, Erlangen). The plasmids were transformed by electroporation (Gene-Pulser, Bio-Rad) into electrocompetent *E. coli* cells XL1-Blue (Stratagene, La Jolla, USA). Alternatively, electrocompetent *E. coli* strain TOP10F' (Invitrogen, Leek, Nethelands) was used.

4.3.12 DNA-DNA hybridization (Southern blot analysis)

DNA-DNA hybridization (Southern blot analysis) according to Sambrook & Russel (1999) was accomplished using radioactive α-^{32}P-dATP or α-^{32}P-dCTP. The production of probes was made by means of random priming (usb, Freiburg) or in a PCR reaction with specific primers.

The DNA samples isolated in agarose gel were capillary transferred to the positively charged nylon filter (Biodyne A, Pall, Ann Arbor, MI, USA). The filter was cross-linked under UV radiation with a dose of 1.2 x 105 μJ (UV Stratalinker 2400, Stratagene, Heidelberg). The probe was purified through prespin Mobispin S-300 Column (Mo Bi Tec GmbH, Goettingen). The filter was prehybridized in Hybridization solution supplemented with 100 μg/ml Salmon sperm DNA more than 1 h at 68ºC and afterwards hybridized overnight with the probe at 68ºC, followed by stringent washing at 68ºC, first, 1 time in 2 x SSC / 0.1% SDS for 10 min, and then 2 times for each 10 min in 0.1-0.2 x SSC / 0.1% SDS. The detection was carried out by means of autoradiography using the films from Kodak (Rochester, NY, USA) or Fuji (New RX, Fuji, Japan). If the filter was reused, a process of stripping was carried out in 0.5% SDS at 95ºC for 2-4 times. The stripping result was radioactively checked.

Hybridization solution	5 x SSC; 1% skim milk; 0.1% Lauroylsarcocine sodium salt; 0.02% SDS
Acidic solution	0.25 M HCl
Denaturation solution	100 g NaOH; 438.3 g NaCl in 5 liter
Netralization solution	242 g Tris; 347 g NaCl in 4 l; pH 7.2
20 x SSC	441.3 g Na_3Citrate; 876.3 g NaCl in 5 l; pH 7.0
2 x Wash	100 ml 20 x SSC; 10 ml 10% SDS in 1 liter
0.2 x Wash	10 ml 20 x SSC; 10 ml 10% SDS in 1 liter

4.3.13 Isolation of total RNA from *A. nidulans*

For isolation of RNA in development stages, approximately 10^3 spores per 9 cm plate was inoculated onto complete medium plates covered with sterile preserving membrane (Ostmann, Bielefeld). Alternatively, a 500 ml CM liquid culture inoculated by spore suspension from one plate was shaken at 200 rpm for 14 h at 37ºC, 50 ml of liquid culture was vacuum filtered through miracloth, the filtered mycelium was put on CM agar plates, incubated at 37ºC. Then, the mycelium together with membrane or miracloth was harvested at defined times, removed extra water between paper towels, frozen in liquid nitrogen and grinded in a mortar. RNA isolation from grinded mycelium powder was carried out with TRIZOL (Gibco or Invitrogen) according to manufacturer protocol. The RNA was finally dissolved in 40-50 μl sterile DEPC H_2O with 0.5 U/μl RNase inhibitor (Promega, Mannheim). The RNA concentration was measured in a

spectrophotometer (Pharmacia LKB, UltrospecIII). The RNA samples were diluted to 2 μg/μl with DEPC H_2O containing RNase inhibitor and kept at –80°C.

4.3.14 DNA-RNA hybridization (Northern blot analysis)

DNA-RNA hybridization (Northern blot) was accomplished as described (Sambrook & Russel, 1999). The RNA was denatured with formamide and separated in denaturing formaldehyde agarose gel, followed by capillary transfer to a positively charged nylon membrane (Biodyne Plus, Pall, Ann Arbor, MI, USA). For size estimation, RNA marker of Promega company (Mannheim) was used. The filter was cross-linked as for Southern blots. Then, the membrane was stained by Methylene blue and washed with H_2O. The two clear rRNA bands should appear. The picture was taken via a camera (INTAS, Goettingen) and video printer (Ann Arbor, MI, USA). The filter was destained in Destaining solution. The filter was prehybridized in Northern hybridization solution with 100 μg/ml Salmon sperm DNA more than 1 h at 42°C and afterwards hybridized overnight with the probe at 42°C, followed by stringent washing at 65-68°C, 1 time in 2 x SSC / 0.1% SDS for 10 min, and then 2 times for each 10 min in 0.5 x SSC / 0.1% SDS. The detection was carried out as in Southern blots.

DEPC water	0.1% DEPC, stir overnight, autoclave
10 x MOPS	0.4 M MOPS (pH7.0); 0.1 M Sodium acetate; 0.01 M EDTA; autoclave
RNA sample buffer	100 μl Formamide; 38 μl 37% Formaldehyde; 20 μl 10 x MOPS; 42 μl DEPC water; 20 μl RNA loading buffer
RNA loading buffer	80% formamide; 1 mM EDTA; 0.1% Bromphenol blue; 0.1% xylene cyanol
Northern staining solution	0.03% Methylene blue in 0.3 M Na-Acetate
Northern destaining solution	1% SDS; 1 x SSC
100 x Denhardt's solution	10 g Ficoll 400; 10 g polyvinylpyrrolidone; 10 g BSA (Pentax fraction V); H_2O to 500 ml, filter and store at –20°C in 25 ml aliquots
Prehybridization / Hybridization solution	5 x SSC; 1% SDS; 5 x Denhardts; 50% formamide

Northern running buffer	100 ml 10 x MOPS, 20 ml 37% Formaldehyde, 880 ml DEPC water
Northern mini gel	0.36 g Agarose, 21 ml DEPC water; boiling, after reaching 70°C; add 6 ml 37% formaldehyde, 3 ml 10 x MOPS

4.3.15 Construction of DNA plasmids

Cloning of the *mutA* gene

The partial sequence obtained from the SSH library (Scherer, 2001) was used to design primers for amplification of a specific mutanase fragment (Glucan1, Glucan2). This fragment was used to identify two cosmids carrying the entire gene from a cosmid library (PUI). From one cosmid (pHG2), a 7.3-kb mutanase-containing *Eco*RV restriction fragment was subcloned into the pCR®-Blunt vector (Invitrogen) (pHGE5).

Cloning of the *mutA* disruption construct (pMut-arg)

Plasmid pHGE5 was cut with *Sac*I and religated in order to remove a *Bam*HI restriction site in the polylinker of the plasmid and one close to the 5'-end of the *Eco*RV fragment (for scheme see result Fig. 5.3). Within this plasmid (pHGE5-Sac) a 0.6-kb *Bam*HI fragment was replaced by *argB* from plasmid pHW-arg, which was constructed by cloning *Kpn*I-*Xho*I released *argB* from pDC1 into pBluescript. The final plasmid was cut with *Kpn*I and *Xba*I, which releases the entire construct plus some vector border sequences, and transformed into *A. nidulans* RMSO11.

Cloning of the *mutA* overexpression construct (pMut-gpd)

The *mutA* open reading frame was amplified with Glucan-ex1 and Glucan-ex2 using the proof reading polymerase Pfu (Promega, Mannheim) and pHGE5 as a template. The PCR product was cloned blunt-end into pBluescirpt KS$^-$ *Eco*RV. The insert was released with *Eco*RI and *Xho*I and inserted behind the *gpd* promoter in pMS-gpd.

Construction of the *mutA::sgfp* transcriptional fusion

The putative *mutA* promoter region (1.8-kb) was amplified by PCR with glu-P1 and glu-P2 using Pfu polymerase. The PCR fragment was cloned blunt end into pBluescript *Eco*RV. The glu-P2 primer introduced a *Not*I restriction site at the start codon of the mutanase. This *Not*I site

and the one in the pBluescript polylinker were used to insert sGFP as a *Not*I fragment obtained from pRF917. Translation of the construct will be terminated by a stop codon within the polylinker after the *sgfp*.

Cloning of the *hgtA* gene

The *hgtA* sequence obtained from *A. nidulans* genomic database (Cereon, USA) was used to design primers for amplification of a specific *hgtA* fragment (HexA5'/3' and HexB5'/3'). This fragment was used to identify a cosmid carrying the entire gene from a cosmid library (PUI) as described for the *mutA* gene. From this cosmid (pH38E7), a 4-kb *hgtA*-containing *Hind*III and a 3-kb *Sal*I-*Pst*I restriction fragments were respectively subcloned into the pCR2.1TOPO vector (Invitrogen) (pHHH1) and pBluescript KS (pHHPS4).

Cloning of the *hgtA* deletion

The fragment containing partial *hgtA* from pHHPS4 was cut by *Sma*I-*Sal*I and inserted *Eco*RV-*Xho*I of pCR2.1TOPO vector. The fragment was released by *Eco*RI-*Xha*I and inserted into *Eco*RI-*Xha*I of pHHH1, in which the *argB* released from pDC1 by *Eco*RI was inserted *Eco*RI site, leading to the *hgtA* deletion construct (pHHRarg11). The final plasmid was linearized by *Xha*I, and transformed into *A. nidulans* wild type strain SRF200 (also see Fig. 5.7 for procedure).

Construction of the *hgtA::sgfp* transcriptional fusion

The putative *hgtA* promoter region (2.5-kb) from the plasmid pHHPS4 was amplified by PCR with KS-Rev1 and Hex-Not1 using Pfu polymerase. The PCR fragment was cloned blunt end into pBluescript *Eco*RV. The Hex-Not1 primer introduced a *Not*I restriction site at the start codon of the *hgtA*. This *Not*I site and the one in the pBluescript polylinker were used to insert *sgfp* as a *Not*I fragment obtained from pMut-p-gfp. Translation of the construct will be terminated by a stop codon within the polylinker after the *sgfp*.

Construction of 5' deletion of *mutA* promoter

The construct pMut-p-sgfp was used as the template for a PCR to amplify *mutA* promoter fragments together with the *sgfp* reporter gene. Here used the different 5' primers corresponding -1.6, -1.45, -1.38, -1.0, -0.9 and -0.75 kb positions in *mutA* promoter and a 3' primer KS-gfp

under the downstream of *sgfp* gene in the polylinker of pBluescript KS$^-$ (see Table 3.9 for primers). The amplified fragments were inserted into the *Eco*RV site of pBluescript KS$^-$. The constructs with *argB*-carrying plasmid pDC1 was used for co-transformation with 5' progressive deletion construct into *A. nidulans* wild type strain RMSO11.

Cloning of the *steC* gene

The partial *steC* sequence upstream of *digA* was used to probe different restriction enzyme-cut cosmid (pAG1) by Southern blotting. A 8 kb *Sal*I-*Kpn*I restriction fragment containing the entire *steC* gene and 4-5 kb upstream and 1 kb downstream of *steC* was subcloned into *Xho*I-*Kpn*I of PCR2.1-TOPO vector (Invitrogen; pHSKS2).

Cloning of the *ste*C disruption construct (pHSAB3, 5)

In plasmid pHSKS2, a 0.6 kb *Bam*HI fragment in *steC* was replaced by *argB* from plasmid pHW-arg. The final plasmid was linearized with *Kpn*I, and transformed into *A. nidulans* wild type strains RMSO11 and SRF200.

Cloning of the *ste*C overexpression constructs

*Pvu*II-*Kpn*I, *Eco*RI-*Kpn*I, *Sac*I-*Kpn*I and *Stu*I-*Kpn*I fragments from pHSKS2, which contain whole or partial sequence of the *steC* open reading frame, were respectively inserted behind the *gpd* promoter in pMS-gpd.

Construction of the *steC::sgfp* transcriptional fusion

A 4 kb *Eco*RV-*Bam*HI fragment containing the putative *steC* promoter region and 2/3 of *steC* ORF was inserted into pBluescript. The *Not*I site in the pBluescript polylinker was used to insert *sgfp* as a *Not*I fragment obtained from pRF917. Translation of the construct will be terminated by a stop codon within the polylinker after the *sgfp*.

4.4 Biochemical methods

4.4.1 Isolation of protein from *A. nidulans*

Mycelium was harvested, dried and powered in a mortar in liquid nitrogen. 2 x Laemmli sample buffer was added into the grinded powder of samples and completely mixed. The reaction

tubes containing samples were put into boiling water or heated at 95˚C for 5-10 min and then cooled on ice, followed by strong vortex and centrifugation at 10000 rpm for 5-10 min. The supernatants were transferred into new tubes and kept at –20˚C.

2 x Laemmli sample buffer	100 mM Tris-HCl (pH 6.8); 4% SDS; 20% Glycerol; 100 mM DTT

4.4.2 Determination of protein concentration (Bradford Assay)

Protein concentration was determined according to Bradford (Bradford, 1976) using the Bio-Rad protein assay (Bio-Rad, Munich). The measurement is based upon Coomassie® Brilliant Blue G-250 dye-binding assay. Acryl-cuvettes (Sarstedt, Nümbrecht) were used for the determination of protein concentration. 200 µl Bio-Rad Protein Dye (Bio-Rad) were added into samples and standard (BSA) (0-50 µg / 0.8 ml H_2O with diluted sample isolation buffer), and well but gently mixed to avoid bubbles. After 5 min, the measurement was carried out in the spectrophotometer (Pharmacia LKB, UltrospecIII) at 595 nm.

4.4.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE gel consisted of a resolving gel topped by a stacking gel. The seperating gel was casted between the glass plates using Bio-Rad Mini Protean II equipment and overlayed by isopropanol. After gel polymerisation, the isopropnol was removed and the gel chamber was filled up with stacking gel and a comb was inserted. The protein samples were diluted to appropriate concentrations using 2 x Laemmli sample buffer, heated at 95ºC for 5-10 min and the samples were loaded onto the gel. Electrophoresis was accomplished at room temperature first with 50 V until the samples moved out of the sample wells and then 100-120 V until the tracking dye reached the bottom of the separating gel.

	Stacking gel	Separating gel		
	4%	6%	10%	12%
Acrylamid / Bisacrylamid 40%	0.36 ml	1.1 ml	1.9 ml	2.25 ml
1 M Tris-HCl pH 6.8	0.45 ml			

1 M Tris-HCl pH 8.8		1.88 ml	1.88 ml	1.88 ml
H_2O	2.8 ml	4.4 ml	3.6 ml	3.25 ml
10% SDS	36 µl	125 µl	125 µl	125 µl
10% APS	15 µl	40 µl	40 µl	40 µl
TEMED	3 µl	5 µl	5 µl	5 µl

10 x Electrophoresis running buffer	30.3 g Tris; 144 g Glycine; 2 g SDS in 1 liter of ddH_2O.

4.4.4 Western blotting

After electrophoresis, the proteins in the gel were transferred to Hybond ECL nitrocellulose membrane (Arersham-Pharmacia Biotech, Freiburg). Electroblotting was performed in a "sandwich" assemble in Transfer buffer for 2 h to overnight at 60 V at 4°C using Mini Trans-Blot Apparatus (Bio-Rad, Munich). After transfer, the membrane was stained for 5 min in Ponceau S solution, then washed using water until the protein bands appeared in the desired way. The membrane was washed in TBST solution 5 x 5 min, blocked in Blocking solution for 1 h, washed again 3 x 5 min in TBST, hybridized overnight at 4°C with the primary antibody in Blocking solution, washed 3 x 5 min in TBST, then, incubated with the second antibody for 1 h at room temperature, followed by 3 x 5 min washing in TBST. The detection was done with the BM chemiluminescence kit from Roche (Mannheim).

10 x Transfer buffer	30.3 g Tris; 144 g Glycine in 1 liter of ddH_2O.
Transfer buffer	800 ml H_2O; 100 ml 10 x Transfer buffer; 200 ml methanol
Ponceau S	0.1% Poncean S in 1% Acetic acid, reusable
10 x TBS	24.2 g Tris; 80 g NaCl in 1 liter of ddH_2O pH 7.6
TBST	1 x TBS; 0.1% Tween 20 (100%)
Blocking solution	TBST with 3% BSA

4.4.5 Preparation of *A. nidulans* nuclear extracts

The mycelium after overnight growth at 37˚C was grinded to a very fine powder. Then 20 ml NIB-A was added. Once a slurry was homogenous, 40 ml NIB-B buffer was added, and then

transferred to a 30 ml Corex tube and centrifuged at 1480 g for 7 min at 4˚C. The supernatant was transferred into a new Corex tube, 5 ml NIB-C buffer was layered, followed by centrifuge at 16500 g for 25 min. The pellet was resuspended in NS buffer or processed as below. The crude nuclear pellet was resuspended in 4 ml of 1 x NEB buffer, and incubated on ice for 30 min with shaking, and then pelleted at 35000 rpm for 30 min. The supernatant was dialysed against 2 x 1 litre of BIND buffer for 4 h at 4˚C. The sample was concentrated using vacuum dialysis or ultrafiltration through a Centriprep 10 unit (Amicon). The protein concentration was determined using Bio-Rad assay.

NIB-A buffer	50 mM Tris-HCl (pH7.5); 5 mM $Mg(C_2H_3O_2)_2.4H_2O$; 20% glycerol; 5 mM EGTA; 3 mM $CaCl_2$; 1M sorbitol; 7% Ficoll
NIB-B buffer	25 mM Tris-HCl (pH7.5); 5 mM $Mg(C_2H_3O_2)_2.4H_2O$; 10% glycerol; 5 mM EGTA;
NIB-C buffer	25 mM Tris-HCl (pH7.5); 5 mM $Mg(C_2H_3O_2)_2.4H_2O$; 10% glycerol; 1 M sucrose
5 x NEB buffer	75 mM HEPES-KOH (pH7.6); 0.5 mM EGTA; 25 mM $MgCl_2$; 25% glycerol; 2 M KCl
NS buffer	25 mM Tris-HCl (pH7.5); 5 mM $Mg(C_2H_3O_2)_2.4H_2O$; 25% glycerol; 0.1 mM Na_2EDTA
5 x BIND buffer	125 mM HEPES-KOH (pH7.6); 200 mM KCl; 5 mM Na_2EDTA; 50% glycerol

All the buffers were sterilized by autoclaving and stored at 4˚C, immediately prior to use, 5 mM DTT and proteinase inhibitor cocktail (Sigma) were added

4.4.6 Purification of DNA-binding proteins

The method was adapted from Joern Kalinowski (University of Bielefeld). Standard PCR amplified 150-200 bp DNA fragments of the promoter with 20-25 bp primers and one of the primers was modified with biotin at the 5'-end. 200-400 µl of Streptavidin agarose was washed 3-5 times with 1 x DNA binding buffer, resuspended in 400 µl 1 x DNA binding buffer. The purified PCR product (about 2 µg / 200 µl) was mixed with 2 x DNA binding buffer (1:1) and 400 µl Streptavidin agarose was added, and then incubated several hours or overnight at room temperature (PCR product binds with biotin to Streptavidin), followed by washing 2-3 times with 1 x DNA binding buffer and then equilibrated with 1 ml protein binding buffer for 30 min. PCR

fragment bound to Streptavidin was incubated with nuclear extract for 10-15 min, followed by washing with 1 ml protein binding buffer. 10-20 µl protein elution buffer was added. The pellet was vortexed carefully and placed on ice for 5 min. After centrifugation, the supernatant was transferred into a new tube and SDS-loading buffer was added to run a SDS-PAGE gel. The gel was stained by silver staining. The separated bands were eluted and digested for MALDI-TOF Mass Spectrum analysis. A PCR fragment of nonspecific binding of transcription factors was used as a control.

2 x DNA binding buffer	10 mM Tris-HCl; 1 mM EDTA; 2 M NaCl; pH 7.5
Protein binding buffer	20 mM Tris-HCl; 1 mM EDTA; 1 mM DTT; 100 mM NaCl; 10% Glycerine; 0.05% Triton X 100; pH 8.0
Protein elution buffer	20 mM Tris-HCl; 1 mM EDTA; 1 mM DTT; 1 M NaCl; 10% Glycerine; 0.05% Triton X 100; pH 8.0

4.5 Fluorescence microscopy

sGFP-expressing strains were observed with a Zeiss Axiophot microscope and appropriate filter combinations. For the documentation a Seascan CCD camera (INTAS, Goettingen) was used.

4.6 Other methods

4.6.1 Quantification of mutan (alkali-soluble fraction)

For each time point 6 agar plates (9 cm diameter) were inoculated and incubated at 37°C. Development was followed microscopically. Mycelium was harvested and the alkali-soluble fraction isolated as described (Zonneveld, 1973). At different time points, the mycelium from 6 plates (9 cm diameter) on minimal solid medium was harvested and then poured into 2 liters of boiling water. After the agar was solubilized, the mycelium mats were washed in fresh boiling water and dried at 80˚C. The dried material was weighed and inoculated with 5% KOH (100 ml/g dry mycelium) for 18 h with shaking at 37˚C, after which it was centrifuged and washed again with 5% KOH. The remaining pellet after centrifugation was called the alkali-insoluble fraction.

The alkaline extracts were neutralized with glacial acetic acid; the resulting flocculent precipitate was centrifuged, washed three times with water, and dried. This alkali-soluble fraction contained the α-1,3-glucan (mutan).

4.6.2 Growth of *Δmut*A, wild type and *mutA* overexpression strains in mutan medium

The alkali-soluble fraction (mutan) was used as sole carbon source. Approximately 1% alkali-soluble fraction from 12 d growth of *ΔmutA* strain (SHW1) mixed with other minimal medium components except glucose was used to pour agar plates. Spore suspensions of *Δmut*A, wild type and *mut*A overexpression strains were inoculated at the center of the agar plate. The plates were incubated at 37ºC and the phenotypes were investigated microscopically.

5 Results

5.1 Analysis of the carbon cycle during sexual development in *A. nidulans*

5.1.1 Molecular cloning of the *mutA* gene

A partial sequence (350 bp) obtained in a substractive hybridization (SSH) library specific for sexual development (Scherer, 2001) encoded a peptide with high homology to fungal α-1,3 glucanases (= mutanase). The partial cDNA sequence was used as a probe to isolate two corresponding cosmids from the PUI library (kindly provided by B. Miller, Idaho, USA). From one of the cosmids (pHG2), a 7.3 kb *Eco*RV restriction fragment was identified by Southern blot containing the mutanase gene (Fig.5.1). It was subcloned (pHGE5) and sequenced (Fig.5.1). The genomic sequence revealed that the mutanase gene was located in a region close to the 3' end of the *Eco*RV restriction fragment. This area, spanning from the *Bgl*II restriction site at about 4000 bp to the *Eco*RV site at the 3'-end, was sequenced on both strands. In order to deduce a putative mutanase protein sequence, a corresponding cDNA was sequenced, which was identified in the EST database (clone j9c02) at the Fungal Genetics Stock Centre (FGSC, http://www.fgsc.net). Comparison of the genomic DNA with the cDNA sequence revealed the presence of three short introns, 48, 52 and 45 bp in length. One is located at the 5'-end of the gene and two in the middle of the coding region. Interestingly, the positions of the first and the second introns are conserved in comparison to the mutanase from *Penicillium purpurogenum* (Fuglsang et al., 2000). The polyadenylation site was derived from the cDNA sequence 50 bp downstream of the stop codon and is also indicated in Fig. 5.1. A putative promoter was identified using a promoter prediction program (http://www.fruitfly.org/cgi-bin/seq:tools/promoter.pl), which determined the start of transcription 54 bp upstream of the start of translation. A putative TATA box motif is located 32 bp upstream of the transcriptional start site. After removal of the intron sequences, an open reading frame of 431 amino acids could be deduced, which encodes a putative protein with a calculated molecular mass of 48 kDa. The protein is hydrophilic with a calculated isoelectric point of 4.6. However, at the N-terminus a 22 amino acid long hydrophobic signal peptide was detected (http://www.cbs.dtu.dk). This suggests that the mutanase protein is secreted. Several short (10 amino acids) stretches of hydrophobic residues are found throughout the sequence and a 50 amino acid long area at the C-terminus of the protein. Pairwise comparison with other fungal

mutanases revealed identities between 24 and 42 % to the *Neurospora crassa, P. pupurogenum, T. harzianum,* and *Schizosaccharomyces pombe* proteins (Fig.5.2). The sequence of *A. nidulans mutA* was deposited in the EMBL database and is available under the accession number AJ310441.

Fig. 5.1 Sequence of *A. nidulans mutA*. **(A)** Scheme of the *mutA* gene locus. **(B)** The *Bgl*II (with asterisk)-*Eco*RV restriction fragment covering the *mutA* gene was sequenced on both strands. The predicted start of transcription is indicated in the promoter region and the polyadenylation site found in one cDNA is indicated both with an asterisk above the sequence. The predicted N-terminal secretion signal is shadowed. The *Bam*HI sites used for the construction of the disruption allele are boxed (see below). DNA and protein analysis was performed with the DNAStar program. The sequence of *A. nidulans mutA* is available under the accession number AJ291452.

Fig. 5.2 Alignment of *A. nidulans* MutA with homologous sequences of *N. crassa* (Q9P563), *P. pupurogenum* (Q9P8T5), *T. harcianum* (AF214480) and *S. pombe* (O13716 and O94510). If amino acids were identical in two or more sequences they were shaded. The alignment was done with DNAStar using Megalign (Clustal) with a gap penalty and a gap length penalty of 10. The putative mutan binding domains from *P. purpurogenum* and *T. harcianum* are indicated with two asterisks above the corresponding amino acids.

5.1.2 *mutA* disruption and overexpression

To gain insights into the biochemical function of MutA in *A. nidulans,* a null mutant was constructed, in which part of the open reading frame of *mutA* was substituted with the nutritional marker gene *argB* (Fig. 5.3; see Methods 4.3.15). Colony-purified arginine-prototrophic transformants were tested by Southern blot for homologous replacement of the *mutA* gene using different restriction digests (Fig. 5.3). In 5 of 46 strains the observed bands indicated disruption of the wild type copy. One strain contained additional integrations of the knock-out construct and was therefore discarded. To show linkage between the disruption event and the nutritional marker gene *argB,* two *mutA* disruption strains (SHW1 and SHW44) were crossed to SRF200 and progeny strains analysed for the *mutA* deletion. Twenty *argB*$^+$ strains were tested in a Southern blot. They all displayed the banding pattern of the deletion. Two strains (SHW1 and SHW44-13) were selected for phenotypic characterisation and comparison with *mutA* wild type strains (SHW26 and SHW29) in minimal and complete medium. No difference with regards to vegetative growth, asexual development or sexual fruiting body formation was found. The number of cleistothecia per cm^2, the number of ascospores per cleistothecium and the viability of the ascospores were also similiar. To further characterise the effect of a loss-of-function mutation of *mutA*, the amount of "alkali-soluble material" (this fraction contains mutan) accumulated during growth was determined (see methods 4.6.1; Fig. 5.3). A wild type and a deletion strain were inoculated on minimal media and grown for different times at 37 °C. Under these growth conditions the strains initiated cleistothecia formation after 4 days. The alkali-soluble material (mutan) was isolated as described (Methods 4.6.1). Whereas in wild type the amount of mutan reached a maximum after 4 days, in the deletion strain the mass of mutan increased steadily. The experiment was repeated three times where mycelium after 6 and 12 days was analysed. Generally, the relative amount of mutan in the deletion strain was higher after 12 days than after 6 days. This might be due to degradation of other cell components and the remaining mutan. The absolute values of the mutan fraction differed from experiment to experiment, but the amount of mutan in the wild type after 12 days was always about 50 % of the mutan in the *mutA* mutant. In our experiment, if estimating the absolute amount of mutan as the mutan fraction of *mutA* deletion strain minused by that of *mutA* overexpression strain after longer growth such as 12

days, the mutan occupied around 5-8% of the amount of supplemented glucose in minimal medium.

In addition to the disruption of the gene, the *mutA* gene was overexpressed under the control of the constitutive *gpd* promoter (pMut-gpd) in the wild type RMSO11 (see methods 4.3.15 for cloning). Three strains (SHW-gpd3, 4 and 5), which showed high transcriptional expression levels in liquid culture after 15 h growth at 37 ºC (Fig. 5.3), were analysed for development and mutan degradation. No difference was found with respect to asexual development and the timing of cleistothecium formation but the amount of mutan was slightly lower than in wild type (Fig. 5.3).

The evident difference in mutan depletion among wild type, *ΔmutA* and *mutA* overexpression strains suggested mutan could be degraded by MutA and reultilized as carbon source. To further visualize the function of MutA, two wild type (SHW26, 29), and two *mutA*-deletion (SHW1, 44-13) and two *mutA* overexpression strains (SHW-gpd4, 5) were inoculated at the center of minimal medium plates where only 1% alkali-soluble material (mutan), harvested from a *mutA* deletion strain (SHW1) after 12 d growth, was used as a sole carbon source instead of 2% glucose (method 4.6.2). The result showed the number of conidiophores in wild type and *mutA* overexpression strains were much higher than in *mutA* deletion strains. During the early days, overexpression strains grew much denser than wild type strains (result not shown). After prolonged growth, the density of conidiophores looked similiar between wild type and overexpression strains, however, more cleistothecia appeared in overexpression strains. This result suggested mutan could be degraded by MutA and contributed to sexual development under the normal growth conditions. A sparse growth of the *mutA* deletion strain was probably due to other carbon sources in the alkali-soluble fraction (Fig. 5.3).

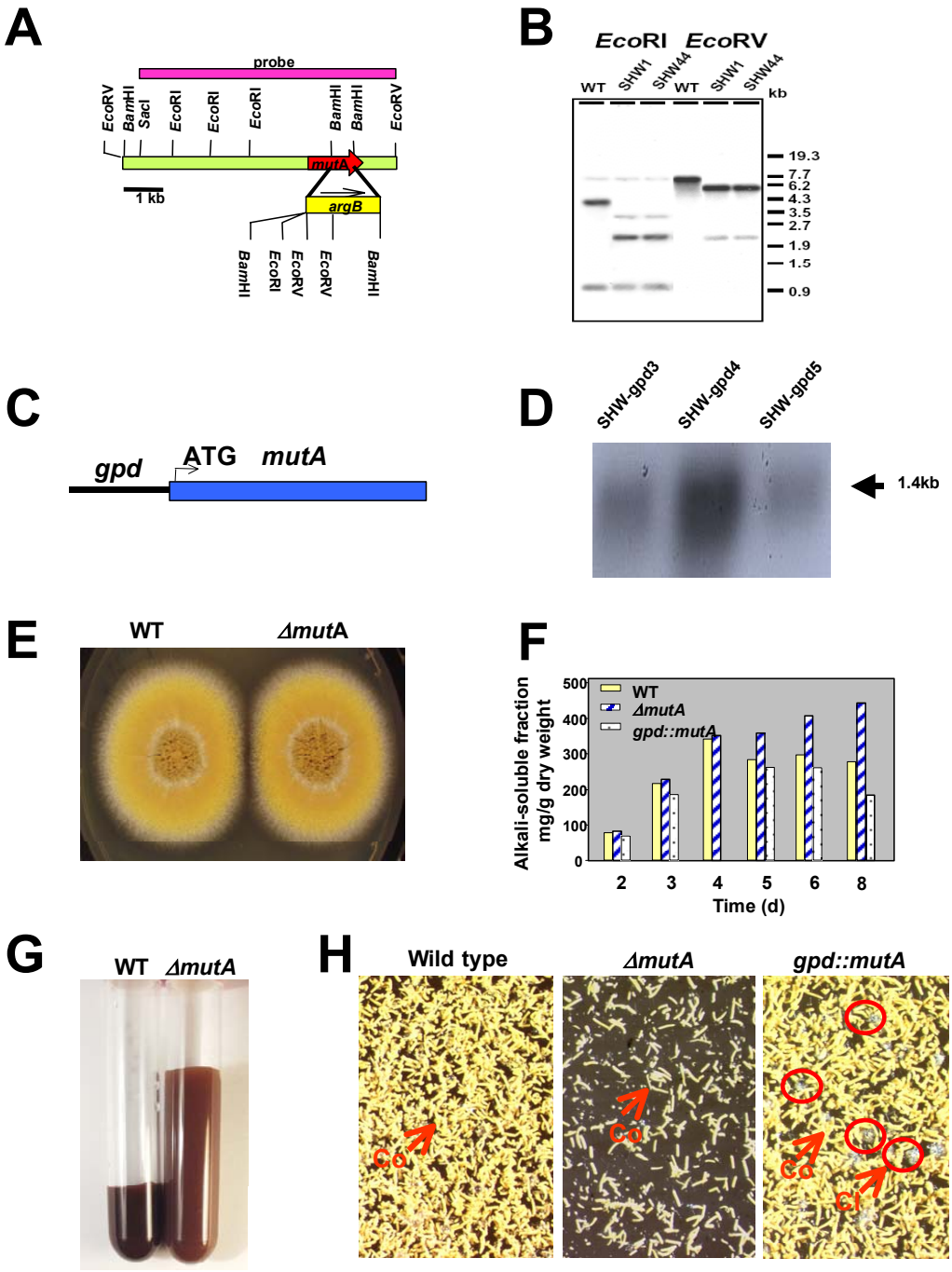

Fig. 5.3 Disruption of the *mutA* gene and overexpression. **(A)** Scheme of the disruption construct (for cloning see Methods 4.3.15). **(B)** Southern blot analysis of *mutA* disruption strains. Genomic DNA of a wild type (RMSO11) and two *mutA* disruption strains (SHW1 and SHW44) was isolated, restricted with *Eco*RI (left panel) and *Eco*RV (right panel), separated on a 1% agarose gel, blotted and hybridised with the probe indicated in (**A**). **(C)** Scheme of constitutive overexpression (see Methods 4.3.15). **(D)** Northern blot analysis of different *mutA* overexpression strains (SHW-gpd3, 4, 5) after overnight growth in liquid culture. **(E)** Conidia-derived colonies 3 days after inoculation onto the

agar plates with complete midium. No difference in vegetative growth, asexual development or later sexual fruiting body formation between a wild type strain (SHW26) and a *ΔmutA* strain (SHW1) was observed. **(F)** Analysis of the accumulation of "alkali-soluble material" (contains mutan) during growth and development in mycelia of a wild type strain (SHW26) (solid bar), a *mutA* mutant (SHW1) (striped bar) and a strain which overexpresses MutA (SHW-gpd4) (stippled bar). For each time point 6 agar plates were inoculated, grown and processed for mutan isolation as described in methods 4.6.1. At time point 4 d the amount was not measured for SHW-gpd4. **(G)** Alkali-soluble material isolated from wild type (SHW26) and SHW1 after 12 days from 6 agar plates. The material was isolated and finally collected in test tubes but not dried as in **(C)**. **(H)** Wild type (SHW26), *ΔmutA* (SHW1) and MutA overexpression strain (SHW-gpd5) were inoculated in minimal agar plates using 1% alkali-soluble fraction (mutan fraction) as sole carbon source instead of glucose, where the mutan fraction was harvested from SHW1 after 12 d growth as in **(F)** and **(G)**. Microscopic observation of colonies was carried out after 7 d growth at 37ºC.

5.1.3 MutA is expressed in Hülle cells

To analyse the spatial expression pattern within the different tissues, the *sgfp* gene was fused to the promoter of the *mutA* gene (pMut-p-sgfp). This construct was co-transformed together with the *argB*-carrying plasmid pDC1 into the arginine-auxotrophic strain RMSO11 and arginine-prototrophic strains were analysed for GFP fluorescence. Two strains (SHW-p-sgfp17 and 19) were further characterised. Bright fluorescence signals were visible on agar plates at hyphal aggregations surrounding cleistothecia. Higher magnification of the areas revealed that Hülle cells and the connecting hyphae showed high expression levels, whereas conidiophores, cleistothecial primordia or ascospores showed no fluoresence (Fig. 5.4). Under growth conditions where no Hülle cells and fruiting bodies were formed, no GFP-fluorescence was observed. The expression pattern resembled the localisation of a catalase peroxidase, CpeA (Scherer et al., 2002).

The obtained results suggested that Hülle cells could act as nurse cells and provide a carbon source to the developing cleistothecium. Hülle cells appear to secrete the a-1,3 glucanase which most likely releases glucose from neighboring hyphae. The question was whether Hülle cells would be also involved in the uptake of glucose. To address this question, a high-affinity hexose transporter was studied.

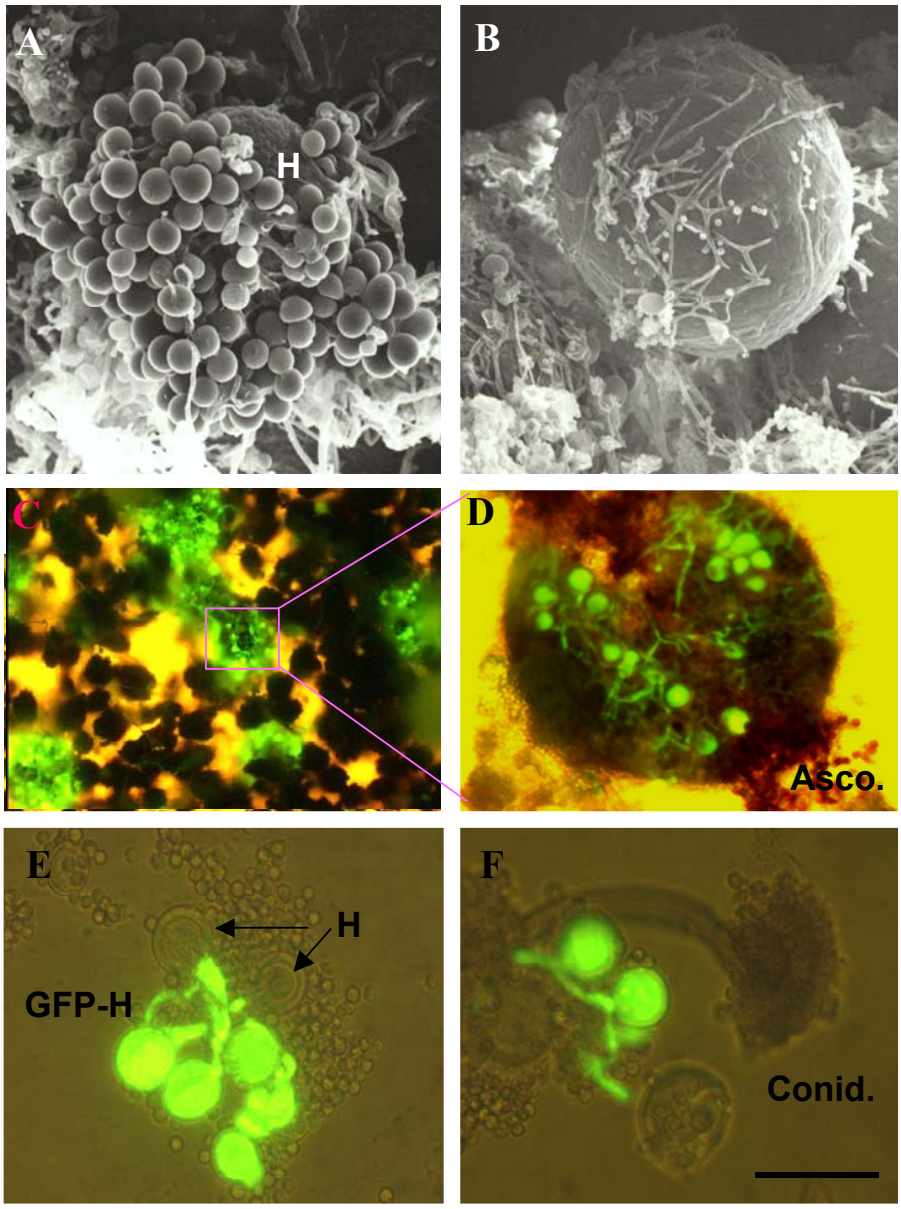

Fig. 5.4. Cellular localisation of *mutA* expression. The *mutA* promoter was fused to *sgfp* and transformed into RMSO11. The strain (SHW-p-sgfp17) was grown on agar plates and sexual development induced. **(A)** Young cleistothecium with Hülle cells (**H**). **(B)** Mature cleistothecium. **(C)** View onto a developing culture. Dark areas are conidiophores, green shining areas are young fruiting bodies. **(D)** One isolated cleistothecium with attached Hülle cells and interconnecting hyphae. Ascospores (Asco.) are not stained. **(E)** Isolated Hülle cells and conidiospores. Some Hülle cells are brightly fluorescent (GFP-H.) whereas others (**H**) are not. **(F)** Brightly stained Hülle cells and non-fluorescent conidiophore (Conid.). The SEM pictures (**A, B**) were taken from Scherer & Fischer (1998). The pictures in (**C, D, E, F**) were taken in dimmed bright field light plus fluorescence light to excite GFP. The scale bar represents 100 µm in (**A, B, D**), 200 µm in (**C**) and 20 µm in (**E, F**).

5.1.4 Molecular cloning of the *hgtA* gene

A partial sequence obtained in the substractive hybridization (SSH) library (Scherer, 2001) encoded a peptide with high homology to a high-affinity hexose transporter gene. The partial sequence information was used to search for corresponding sequences in the Cereon Genomics LLC (Cambridge, MA, USA; http://www.cereon.com) database and a 2.7 kb region was assembled, which covers the entire ORF (Weber, 2002). This gene was named *hgtA*. However, the DNA sequence deposited in the Cereon database could be incorrect in some places. Therefore, it was considered as an appropriate to isolate a genomic DNA clone and resequence the *hgtA* locus. PCR was used to screen the *A. nidulans* PUI cosmid library (see method 4.3.15). A *hgtA*-containing cosmid was identified (pH38E7). From this cosmid, a 3 kb *Sal*I-*Pst*I fragment containing a *hgtA* upstream and partial *hgtA* 5' region and a 4 kb *Hind*III containing full *hgtA* sequence were respectively subcloned (methods 4.3.15). The *hgtA* locus was sequenced and some nucleotide errors removed by comparison with the assembled sequence. The cDNA was generated by reverse transcription-PCR with total RNA from the stage of sexual development as a template for the reverse transcriptase reaction (Weber, 2002). However, the cDNA was incomplete and thus the predicted protein was wrong. Therefore, the cDNA was amplified again using oligonucleotides from further upstream sequences. Sequence comparison revealed an extra intron in the 5' region and revealed an open reading frame of 2 kb interrupted by 6 introns with 44-67 bp in length in the coding region. The translated sequence was predicted to encode a protein of 531 amino acids in length with a molecular weight of 59 kDa. Hydrophobicity values determined at each residue by the Program TMHMM 2.0 (http://www.cbs.dtu.dk) showed the presence of 12 putative transmembrane (TM) domains, a larger cytoplasmatic loop between the sixth and seventh transmembrane helix, a characteristic feature of the major facilitator superfamily, which includes a variety of transport systems in eukaryotes and in prokaryotes (Fig.5.5)(Marger & Saier, 1993).

The HgtA protein shows the high homology to other glucose transporter genes such as glucose transporter (42% identity) in *T. harzianum (*Delgado-Jarana and Benitez, 2000), Hgt1 (37% identity) in *K. lactis* (Billard P. et al., 1996), CaHgt1 (35%) in *C. albicans* (Varma A. et al., 2000), Ght6 (32% identity) in *S. pombe* (Wood V, 2002) and Snf3 (32% identity) in *S. cerevisiae* (Goffeau A., 1996). The alignment revealed a conserved signature (CDRFGRRPAILIG) typical

of sugar facilitators (Marger & Saier, 1993). The sequence of *A. nidulans hgtA* was deposited in the EMBL database and is available under the accession number AJ535663.

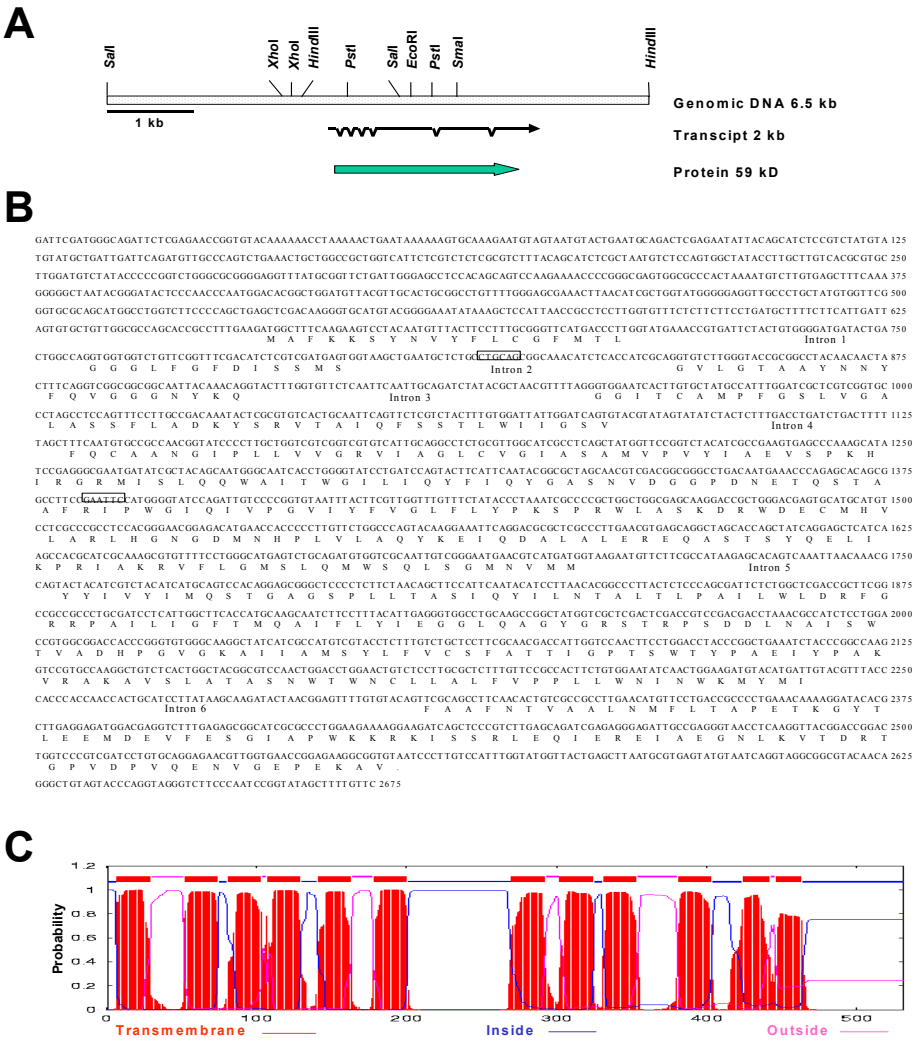

Fig. 5.5 Gene locus and hydrophobicity of *A. nidulans hgtA*. **(A)** Scheme of the *hgtA* gene locus. **(B)** Nucleotide and deduced amino acid sequence of the *hgtA* gene. The positions of six introns were denoted below the DNA sequence. The *Pst*I and *Eco*RI sites used for the construction of the disruption allele are boxed (see below). DNA and protein analysis was performed with the DNAStar program. The sequence of *A. nidulans hgtA* is available under the accession number AJ535663. **(C)** Hydrophobicity plot of HgtA of *A. nidulans*. The plot was constructed from the hydrophobicity value with programm TMHMM 2.0 (http://www.cbs.dtu.dk)

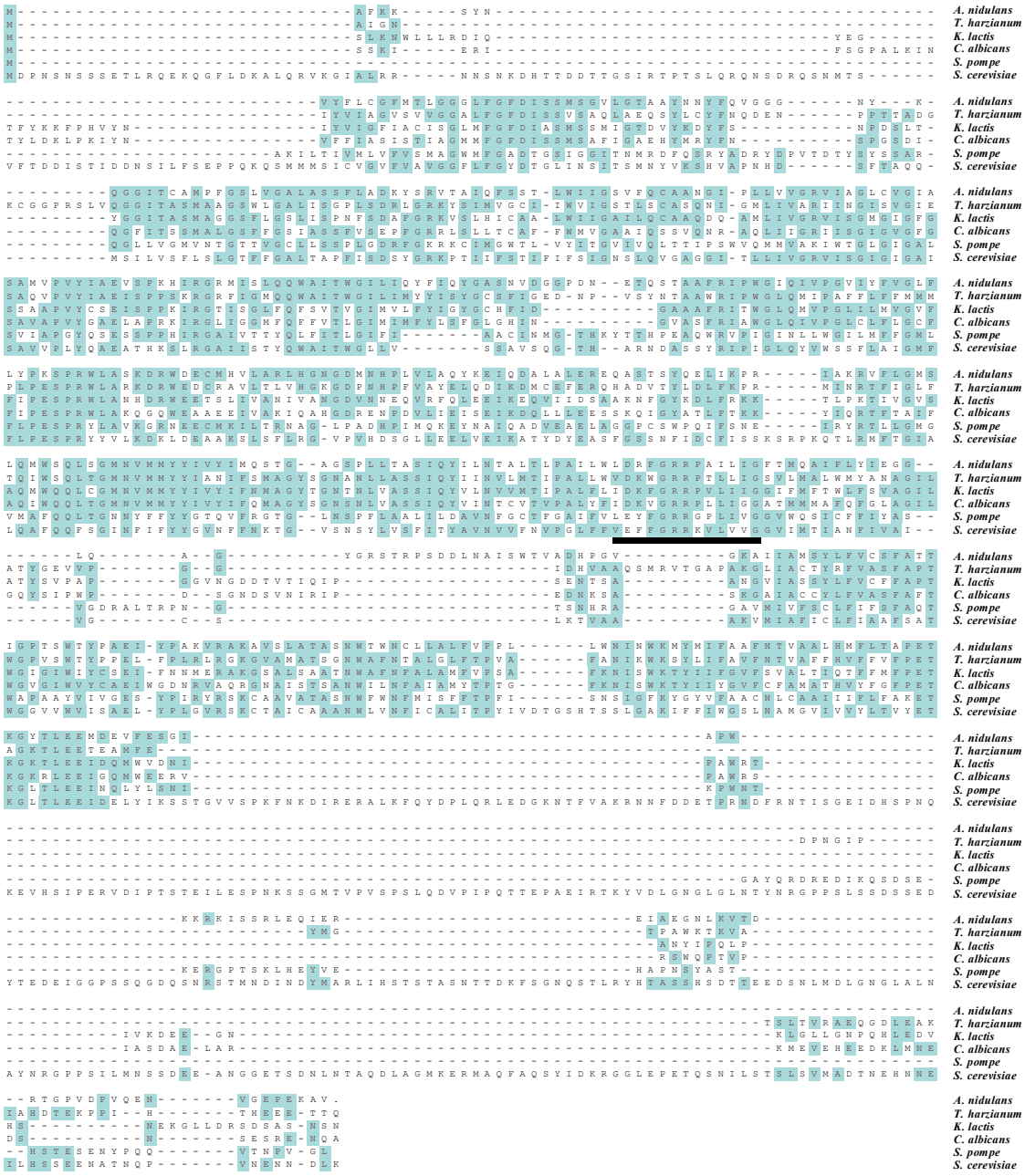

Fig. 5.6 Alignment of *A. nidulans* HgtA with homologous sequences of *T. harcianum* (CAC81782), *K. lactis* (P49374), *C. albicans* (O74713), *S. pombe* (O74849) and *S. cerevisiae* (A31928). If amino acids were identical in two or more sequences they were shaded. A conserved signature (LDRFGRRPAILIG) typical of suger facilitors (Marger & Saier, 1993) was underlined. The alignment was done with DNAStar using Megalign (Clustal) with a gap penalty and a gap length penalty of 10.

5.1.5 *hgtA* disruption

To gain insights into the biochemical function of *hgtA* in *A. nidulans,* a null mutant was constructed. Part of the open reading frame of *hgtA* was substituted with the nutritional marker gene *argB* (Fig. 5.7).

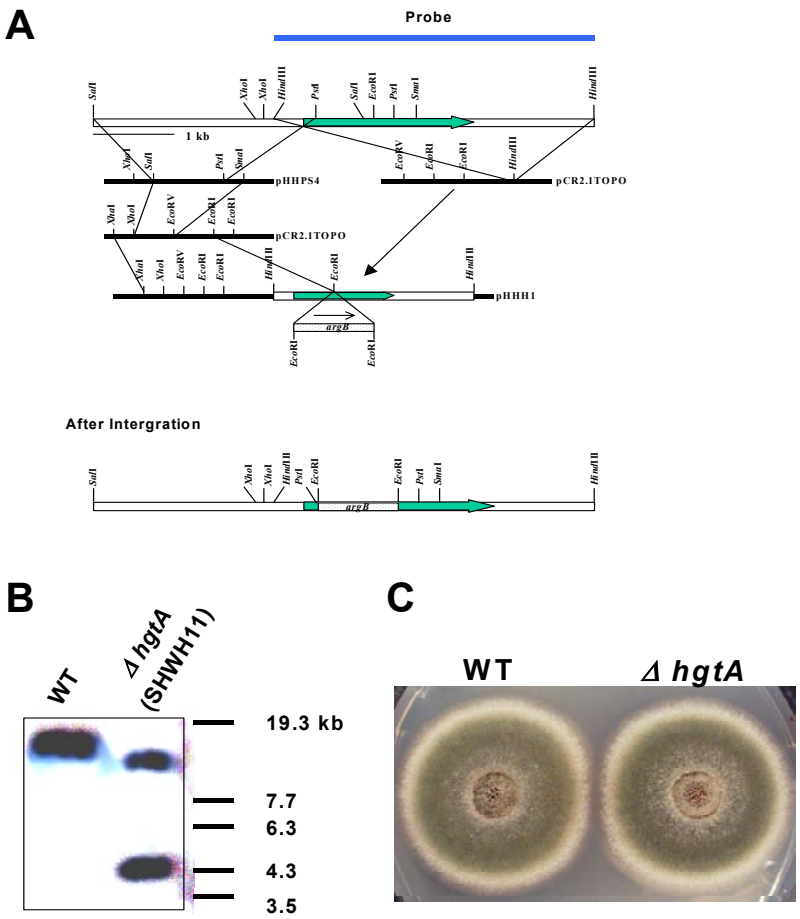

Fig. 5.7 Disruption of the *hgtA* gene. (A) Procedure for construction of the *hgtA* disruption. A 3 kb *Sal*I-*Pst*I fragment of the 5' region of *hgtA* was inserted into pBluescript (pHHPS4), the *Sal*I-*Sma*I released fragment from pHHPS4 was inserted into *Xho*I-*Eco*RV in pCR2.1-TOPO. From this construct, a *Xha*I-*Eco*RI released fragment was inserted into pHHH1, and then, *argB* from pDC1 was inserted into *Eco*RI, leading to the *hgtA* deletion construct, in which a 0.6 kb *Pst*I-*Eco*RI fragment in *hgtA* was replaced by *argB* and some polylinker sequence of the vectors. **(B)** Southern blot analysis of a *hgtA* disruption strain. Genomic DNA of a wild type (SRF200) and a *hgtA* disruption strains (SWHH11) was isolated, restricted with *Bam*HI, separated on a 1% agarose gel, blotted and hybridized with the probe indicated in (**A**). **(C)** Conidia-derived colonies 3 days after inoculation onto agar plates with complete medium. No difference in vegetative growth, asexual development or later sexual fruiting body formation between a wild type strain (SWTB) and a *ΔhgtA* strain (SWHH11) was observed**.**

The deletion construct pHHRarg11 was linearized with *Xba*I and transformed into the *A. nidulans* wild type strain SRF200. The purified arginine-prototrophic transformants were tested by Southern blot for homologous replacement of the *hgtA* gene using different restriction digests (Fig. 5.7). In 1 of 20 strains the bands suggested disruption of the wild type copy (SWHH11; Fig. 5.7). For phenotypic characterisation and comparison with *hgtA* wild type strains (SWTB) in minimal medium and complete medium, no difference with regards to vegetative growth, asexual development or sexual fruiting body formation was found.

5.1.6 *hgtA* is expressed in ascogenous hyphae within cleistothecia

To analyse the spatial expression pattern, the *hgtA* promoter was fused to *sgfp* and co-transformed into *A. nidulans* wild type strain SRF200 with the plasmid pDC1. Green fluorescence appeared on the transformation plate. After purifying, two representative strains (SWHgfp1 and 7) were further characterised. Bright fluorescence signals were visible in swollen hyphal structures, possibly the ascogenous hyphae after dissecting the cleistothecia. Higher magnification of the areas revealed that ascogenous hyphae showed high expression levels. In contrast, conidiophores, Hülle cells, cleistothecial primordia and ascospores showed no fluorescence (Fig. 5.8). In addition, the GFP fluorescence showed changing intensity in different parts of the cells.

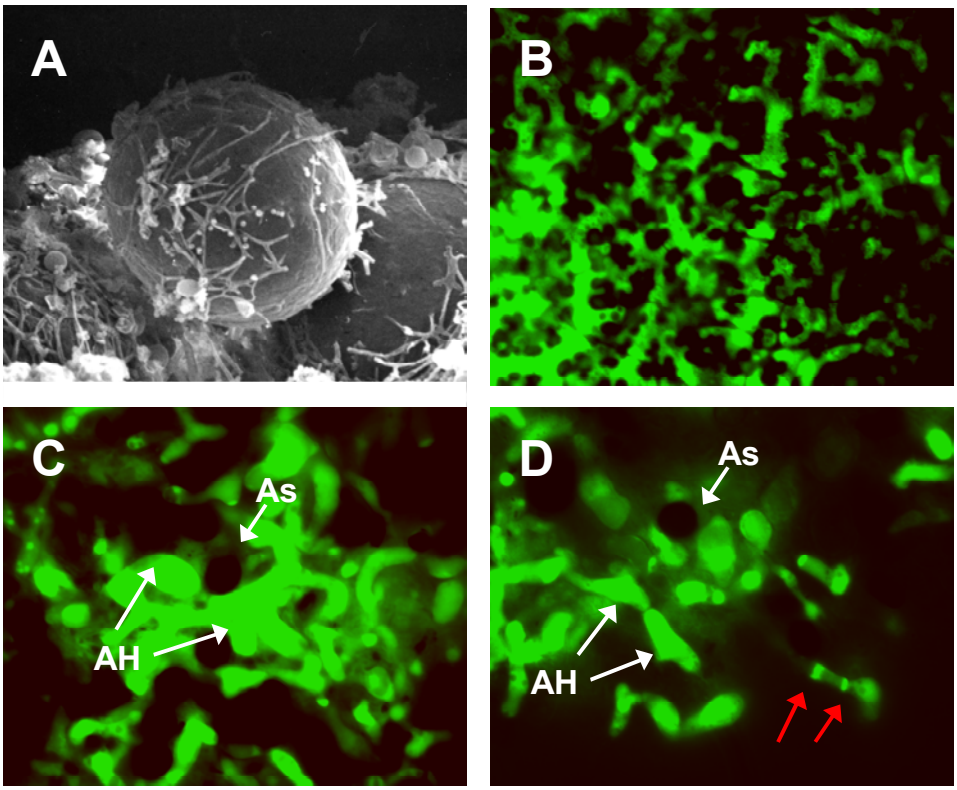

Fig. 5.8. Cellular localisation of *hgtA* expression. The *hgtA* promoter was fused to *sgfp* and transformed into SRF200. **(A)** Mature cleistothecium (Taken from Scherer & Fischer, 1998). **(B)** Green shining on the shell of a young cleistothecium. **(C)** One dissected cleistothecium, bright fluorescence appeared in the swollen hyphal structure, possibly ascogenous hyphae. Ascospores (As) are not stained. **(D)** The GFP fluorescence showed changing intensity in different parts of the cells (red arrows), possibly due to local aggregation of cytoplasm.

These results showed that the α-1,3 glucanase and the high-affinity hexose transporter are expressed in different tissues. The next question was how the specificity of the expression is achieved. To address this problem, the promoter region of *mutA* was characterized, and it was tried to isolate regulatory proteins binding to this promoter.

5.1.7 Identification of regulatory regions in the upstream sequence of *mutA*

To further study the mechanisms underlying the induction of *mutA* expression in Hülle cells, it was sought to locate the DNA-binding elements in the *mutA* promoter involved in the specific induction. A progressive 5'-deletion analysis of the *mutA* promoter was performed to identify *cis*-

acting elements involved in the Hülle cell specific expression. Serially deleted *mutA* promoter fragments fused to *sgfp* reporter gene were produced by PCR (method 4.3.15). The constructs were used to transform *A. nidulans* wild type strain RMSO11. At least 12 randomly picked transformant strains for each promoter fragment were investigated microsopically for green fluorescence intensity. A more quantitive assay using e.g. a spectrophotometer was difficult because of the spatial restriction of the expression in the Hülle cells.

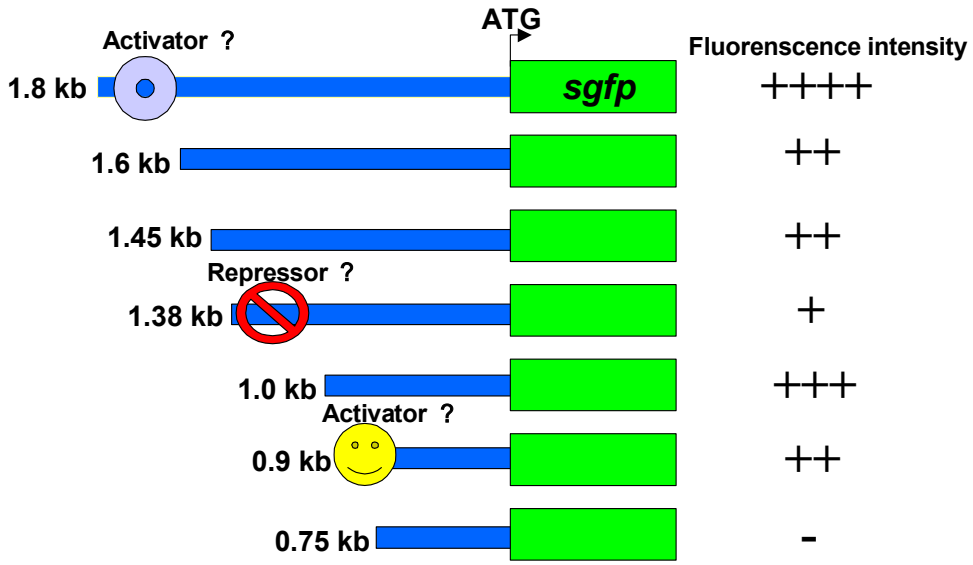

Fig. 5.9 Identification of regulatory regions in the upstream sequence of *mutA*. The progressive 5' deletion fragments of the *mutA* promoter fused with *sgfp* were transformed into *A. nidulans* strain RMSO11. At least 12 transformant strains for each promoter fragment were investigated microscopically for green fluorescence and average values of fluorenscence intensity was estimated. – indicates that no fluorescence could be detected; + to ++++, increasing levels of green fluorescence. Putative repressor and activator sites are labeled.

Deletion of a 420 bp promoter fragment (from -1.38 to -1.8 kb) relative to the *mutA* start codon, caused a decreased fluorescence, but, when the promoter fragment was reduced to the position of -1.0 kb, GFP fluorescence increased again compared with the constructs at -1.38 kb fusion. That suggested at least one putative repressor locates between -1.0 to -1.38 kb. Similarly, at least one putative activator should locate respectively in the regions of -0.75 to -1.0 kb and -1.6 to -1.8 kb (Fig. 5.9). This result suggested that at least three putative binding motifs of transcription factors exist in the *mutA* promoter.

5.1.8 Binding protein isolation

Gel shift is a standard method used for identifying regulatory elements. But, in this study, a new method for isolation of DNA-binding proteins was used (methods 4.4.5; 4.4.6). Wild type strain RMSO11 was grown overnight at 37ºC in 2 liter minimal medium. The harvested mycelium was used to isolate nuclei (methods 4.4.5). To identify potential transcription factors, a 150 bp DNA fragment ranged at –1.7 kb position in the promoter of the *mutA* gene was amplified using primers (glu-P1B/glu-P6F) with one primer modified by biotin at the 5' end, a 170 bp fragment (glu-P4B/glu-P4F) at –1.3 kb in the promoter was amplified as a control. The purified PCR fragments were individually mixed with the nuclear protein from *A. nidulans* (see methods 4.4.6). DNA-protein complexes were loaded onto a 10% SDS-PAGE gel. The gel staining was carried out by silver staining. One protein band with a molecular mass of about 40 kDa was found when the DNA fragment containing the putative binding sites was used, but not with the control DNA (Fig.5.10).

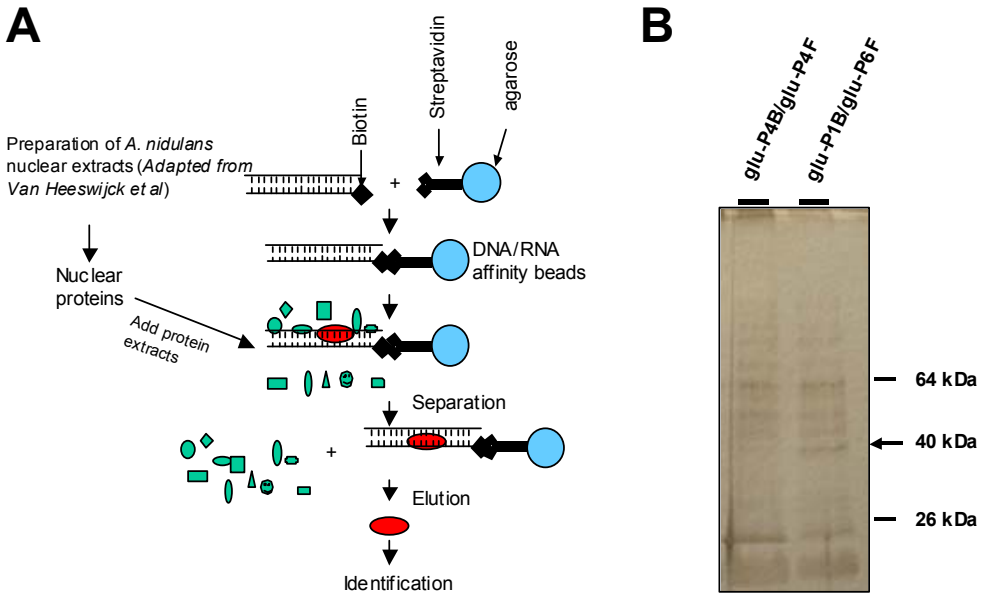

Fig. 5.10 Binding protein isolation in the promoter of *mutA*. **(A)** Procedure of binding protein isolation (see methods 4.4.5; 4.4.6) **(B)** Two fragment amplified by PCR using the primers glu-P4B/glu-P4F; glu-P1B/glu-P6F were used in DNA binding protein assay. A band around 40 kDa appeared to specifically bind to glu-P1B/glu-P6F fragment.

Unfortunately, the identification using peptide mass fingerprinting is not yet established because of the lack of the complete DNA sequence database at the time of investigation. This will be available shortly and then it should be possible to get access to the regulatory proteins.

5.2 Signal transduction in sexual development of *A. nidulans*

5.2.1 Molecular cloning of the *steC* gene

After the characterization of two differentially induced target genes, the role of a MAP kinase cascade as a potential signalling pathway which could be involved in the expression of sexual cycle specific genes was investigated. During the cloning of *digA* (encoding a vesicle sorting protein), a partial open reading frame was identified in the upstream region of *digA* (Geißenhöner *et al.*, 2001). The derived protein sequence (C-terminus) displayed homology to the MAPKK kinases Ste11 from e.g. *S. cerevisiae* and Nrc-1 from *N. crassa*. Because of the similarity to Ste11, the "sterile" phenotype of *steC* deletion mutants (see below), the previous characterization of the homeodomain protein SteA of *A. nidulans* (Vallim et al., 2000), and the description of another gene, *steB*, with similarity to Ste11 (Han & Prade, 2002), the gene was named as *steC*. The partial sequence information was used to search for a corresponding sequences in the Cereon Genomics LLC database and assembled a 4.8 kb region, which covers the entire open reading frame. Restrction analysis and Southern blot analysis of *digA*-containing cosmid pAG1 using *steC* partial open reading frame as a probe indicated that an 8 kb *Sal*I-*Kpn*I contains full *steC* gene (result not shown), that was subcloned (pHSKS2) and sequenced in the *steC* region and some errors in nucleotides were removed by comparing with the assembled sequence from Cereon Genomics LLC. In Northern blot analyses of vegetative cells a transcript of 4 kb with rather low abundance was detected (Fig. 5.18). The cDNA fragments were generated by RT-PCR using different primer pairs and sequenced. Comparison of the cDNA with the genomic sequence revealed the presence of three introns (51, 53 and 49 bp in length) in the 5'-region of the gene (Fig. 5.11).

Fig. 5.11 Sequence of *A. nidulans steC*. A 4.8 kb *steC*-containing region assembled from Cereon Genomics LLC was sequenced again by using the plasmid pHSKS2 as a template. Three introns in the N-terminal were identified by RT-PCR. The deduced amino acid sequence is given below the DNA sequence in the one-letter code. The *Pvu*II, *Eco*RI, *Sac*I and *Stu*I used for overexpression of *steC* or kinase catalytic domain as below were boxed, the corresponding start codon ATG are underlined. The two *Bam*HI restriction sites used for deletion of *steC* were boxed in gray (see below). The concerved SAM domain and the kinase catalytic domain of SteC were shadowed in light yellow. The sequence is available in the EMBL database under the Accession No. AJ505944.

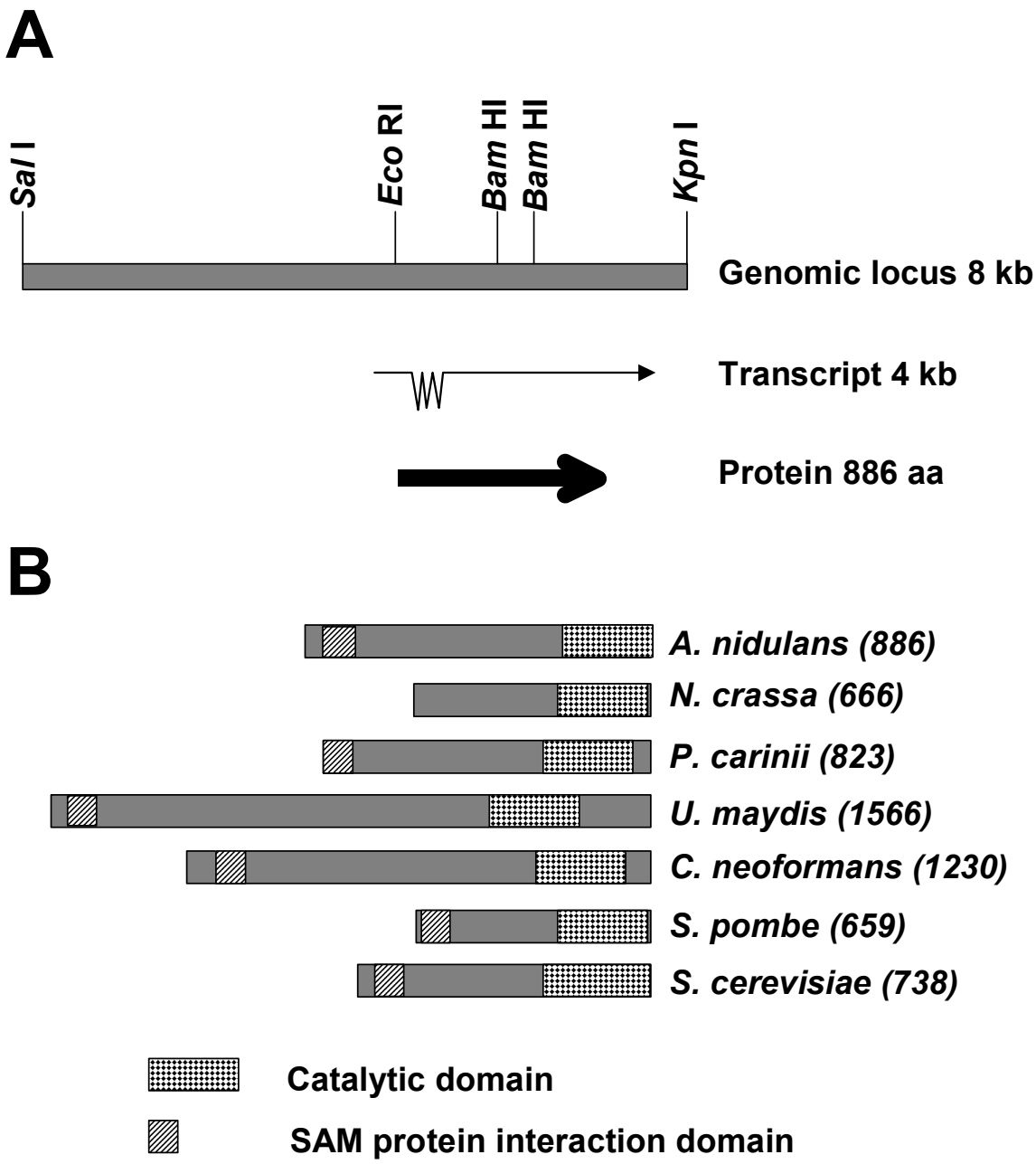

Fig. 5.12 Scheme of *A. nidulans steC*. **(A)** Scheme of the *steC* gene locus, the transcript with the three introns and the derived protein. **(B)** Scheme of the domain organisation of SteC and several homologous proteins from other fungi. The length of the proteins is given in brackets behind the species name. The NCBI accession numbers of the proteins are: *A. nidulans* AJ505944*; N. crassa* AF034090; *P. carini* AF312696; *U. maydis* AF542505.1 (Kpp4=Ubc4); *C. neoformans* AF294841; *S. pombe* M74293; *S. cerevisiae* X53431.

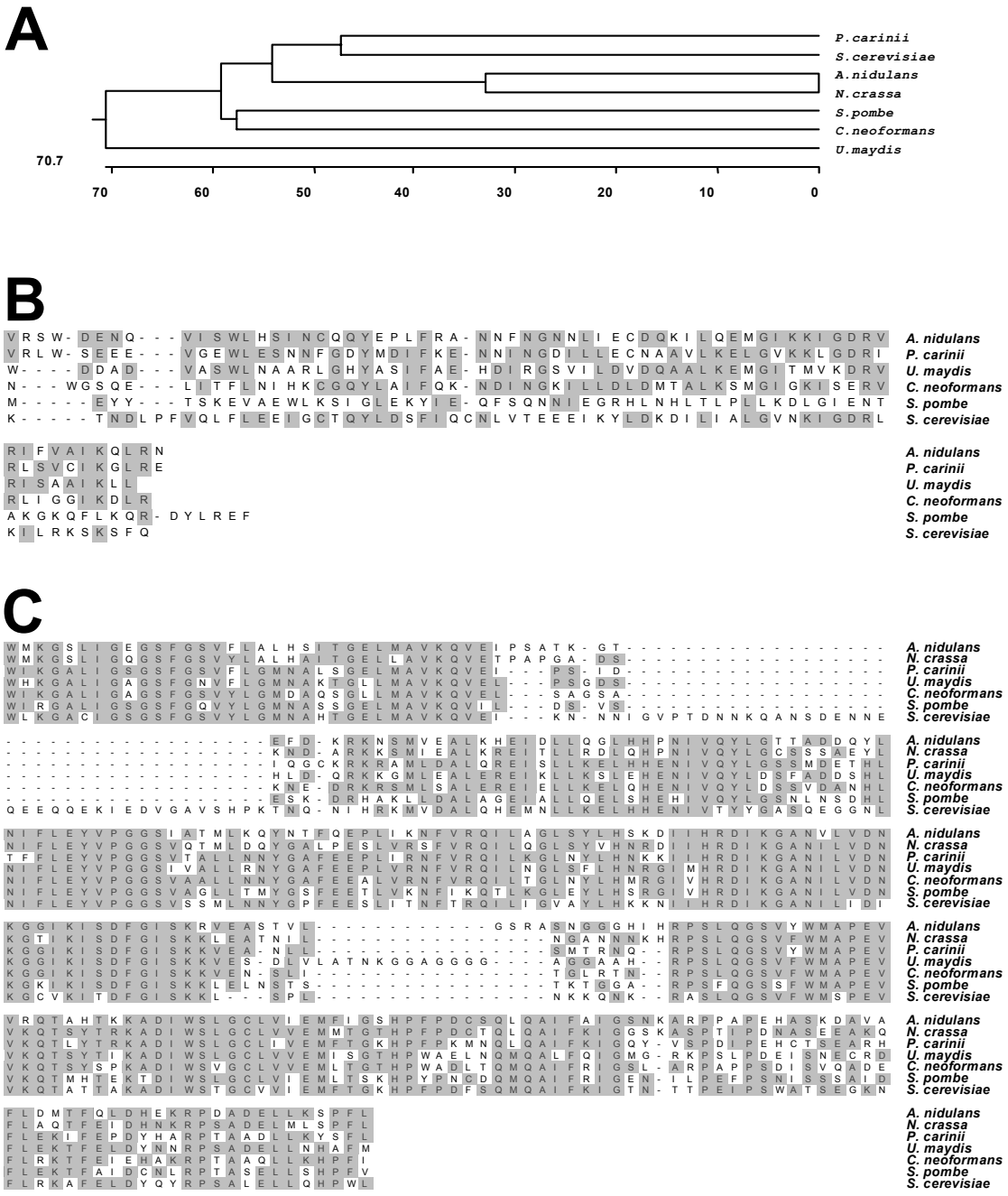

Fig. 5.13 Alignment of *A. nidulans* SteC with homologues from different fungi. Phylogenetic tree of *A. nidulans* SteC showing evolutionary distances from the homologues of *N. crassa* (AF034090); *P. carini* (AF312696); *U. maydis* (AF542505.1); *C. neoformans* (AF294841); *S. pombe* (M74293); *S. cerevisiae* (X53431) **(A)**. The SAM protein interaction domains **(B)** and the catalytic domains **(C)** of the SteC homologues of the species given in Fig. 5.12 were aligned. If amino acids were identical in two or more sequences they were shaded. The alignment was done with DNAStar using Megalign (Clustal) with a gap penalty and a gap length penalty of 10.

After removal of these introns, the translated sequence was predicted to encode a protein of 886 amino acids in length with a molecular weight of 97.8 kDa (Fig. 5.11). The protein shares 50 % identical amino acids with Nrc-1 from *N. crassa* and 32 % (673 amino acids overlap) with Ste11 from *S. cerevisiae* (Chaleff & Tatchell, 1985; Kothe & Free, 1998). The SteC protein sequence contains a SAM protein interaction domain (sterile alpha domain) in the N-terminus and the highly conserved catalytic domain typical of the serine-threonine protein kinase in the very C-terminus (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi). The similarities of the proteins are much higher if only the catalytic domains are compared (Fig. 5.13). The presence and arrangement of the domains is similar to other MAPKK kinases, although the SAM domain is missing in some species (e.g. *N. crassa*) (Fig. 5.12). Amnio acid identity over catalytic domain is 67.5%, 61.1%, 58.2%, 58.5%, 54.3% and 53.5% for *N. crassa, P. carinii*, *U. maydis*, *C. neoformans, S. pombe* and *S. cerevisiae* respectively. As a difference between *S. cerevisiae* Ste11 and the displayed six other fungal MAPKK kinases, Ste11 possesses an extra loop in the catalytic domain. The percentage identity over SteC SAM domain is 48.5%, 41.0%, 39.7%, 24.2%, and 27.7% for *P. carinii*, *U. maydis*, *C. neoformans, S. pombe* and *S. cerevisiae* respectively.

5.2.2 Complementation analysis of *steC* mutant and *steC* overexpression

To overexpress *steC* and its kinase domain, and to address the question whether the SAM domain is necessary for the signalling function of the protein, The *steC* and its N-terminally truncated versions were overexpressed under the control of a constitutive promoter *gpd* and tested them for complementation of the null phenotype, SWH51 (*ΔsteC*, see next chapter) and a wild type strain RMSO11 were respectively transformed with the plasmids pHSKP3 (full length, 886 aa), pHSKR2 (full length, 886 aa), pHSKSa4 (573 aa), pHSKSt4 (459 aa; Fig. 5.14). Overexpression of different fragments was confirmed by Nothern blotting (Fig. 5.14). No evident phenotypes were observed for the overexpression of any fragments. Wild type colonies were obtained only in the case of the full length protein (pHSKP3; pHSKR2) after transformation of steC mutant strain (SWH51). This demonstrates that the loss of 198 amino acids, including the SAM domain, abolishes biological activity and thus suggests that this protein motif is essential for full function.

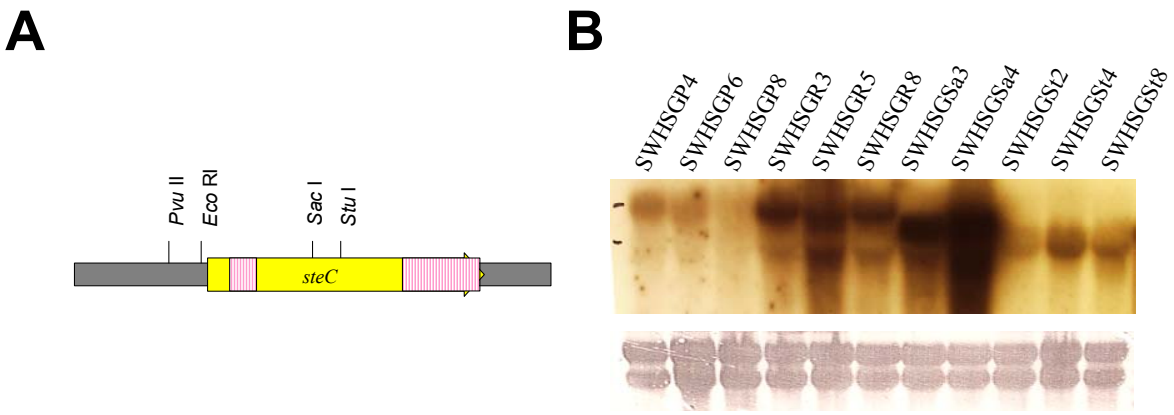

Fig. 5.14 Complemention analysis of *steC* mutant and *steC* overexpression. (A) Different *steC* fragments, two full length clones (*Pvu*II-*Kpn*I fragment pHSKP3, *Eco*RI-*Kpn*I fragment pHSKR2) and two N-terminal truncated fragments (*Sac*I-*Kpn*I fragment pHSKSa4, *Stu*I-*Kpn*I fragment pHSKSt4) were overexpressed under the control of the constitutive promoter *gpd*. The positions of the SAM and the catalytic domains were shaded with vertical lines. **(B)** Northern hybridization of the transformant strains after different *steC* overexpression versions transformed *ΔsteC* strain SWH51. SWHSGP4,6,8 from pHSKP3-transformed SWH51; SWHSGR3,5,8 from pHSKR2; SWHSGSa3,4 from pHSKSa4; SWHSGSt2,4,8 from pHSKSt4. The transformant strains were grown overnight at 37˚C for RNA isolation. As a loading control, ribosomal RNA was stained with methylene blue after transfer to the nylon membrane (low panel).

5.2.3 *steC* deletion affects hyphal growth and conidiophore morphology

To gain insights into the function of the *steC* gene in *A. nidulans,* a deletion strain was constructed. An internal 0.6 kb *Bam*HI fragment was replaced with the nutritional marker *argB* in the 8 kb *Sal*I-*Kpn*I subclone from the *digA* containing cosmid (pAG1; pHSAB3; Fig. 5.15). Integration of the construct through a double crossing-over event leads to the loss of the catalytic domain and disrupts the open reading frame. The construct pHSAB3 was linearized with *Kpn*I and used for transformation of two wild type strains, RMSO11 and SRF200. Transformation yielded wild type-like colonies and about 15 % colonies with a distinct phenotype in orginal transformation plate. Those strains showed a slower growth, the invading hyphae growth into the agar and a darker color bottom of colonies. Southern blot analysis of 18 of those strains revealed that the homologous integration event had taken place (result not shown). Two strains (SWH51 and SWH33) were chosen for further studies (Fig. 5. 15). To confirm that the observed phenotype was due to the predicted integration event, the deletion strain SWH51 was transformed with the

steC carrying plasmid pHSKS2. This plasmid rescued all mutant phenotypes, indicating that it contained the entire *steC* gene and the observed phenotype was due to the disruption of *steC* (Fig. 5.15).

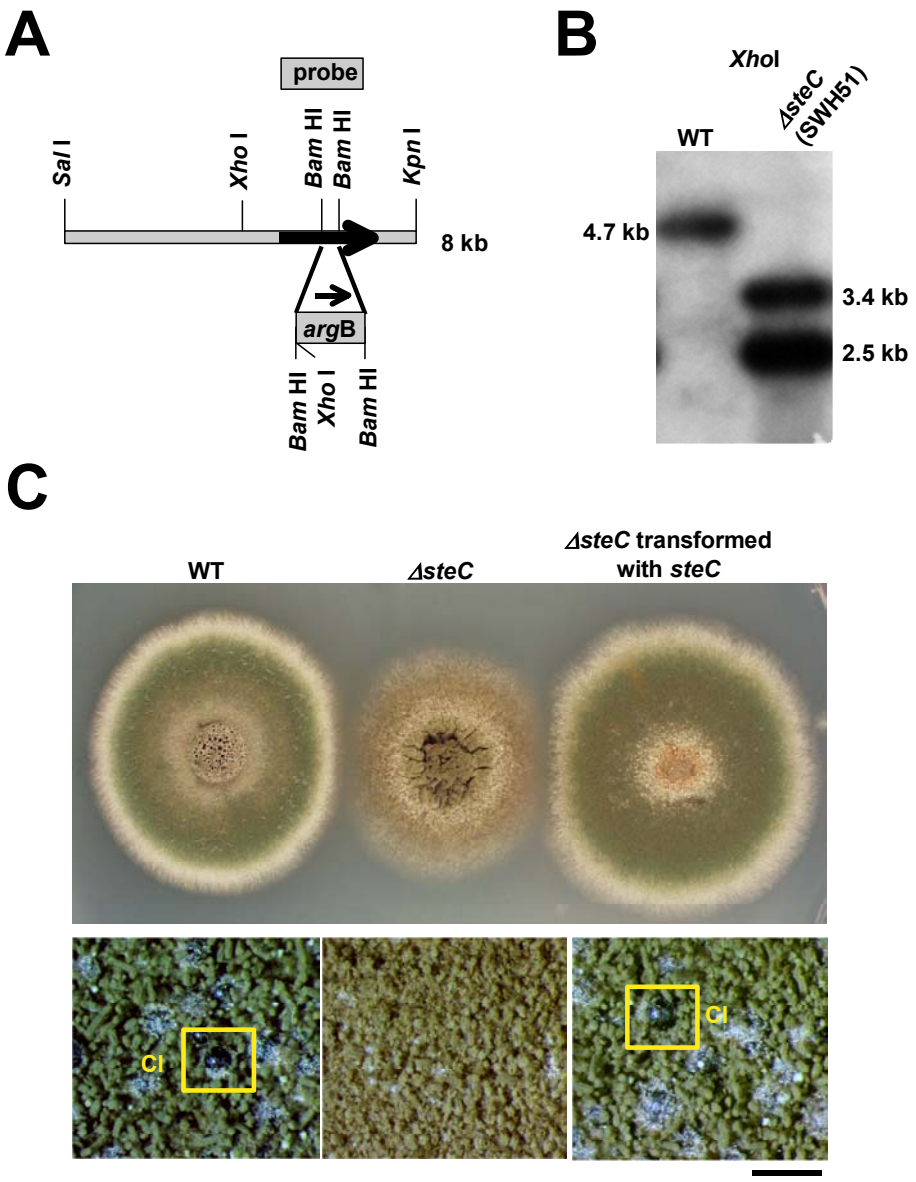

Fig. 5.15 Disruption of the *steC* gene. (A) Scheme of the disruption construct (for cloning see text). **(B)** Southern blot analysis of a *steC* disruption strain. Genomic DNA of a wild type (SRF 200) and *steC* disruption strains (SWH51) were isolated, restricted with *Xho*I, separated on a 1 % agarose gel, blotted and hybridised with the probe indicated in (**A**). **(C)** Colonies of wild type (WT; SWHC18), the *ΔsteC* mutant SWH51 and SWHSR3 (retransformed *steC* mutant by the plasmid pHSKS2) grown on an agar plate supplemented minimal medium and enlargements of the colonies (lower row of pictures) showing conidiophores and cleistothecia (boxed). Scale bar represents 0.5 cm (upper picture) and 1 mm (lower panels).

The *steC* deletion strain was compared to wild type with respect to hyphal growth, hyphal morphology, osmosensitivity, conidiophore development and sexual reproduction. Growth on minimal media resulted in a reduced growth rate and hyphae appeared more curled and more branched than the wild type. Conidiophore development was initiated like in wild type but the height of the stalks was uniform in wild type and varied in the mutant (Fig. 5.16). Metulae and phialides appeared to be normal. However, in a small percentage of conidiophores, the morphology was drastically altered. Only few metulae arose from the vesicle and did not develop according to the normal program. Frequently, secondary conidiophores grew out of the vesicle (Fig. 5.17). In addition, the diameter of the conidiospores varied significantly (Fig. 5.16).

Recently, it was reported that in *A. nidulans* a MAP-kinase cascade is involved in the adaptation of the fungus to high osmotic conditions (Han & Prade, 2002; Kawasaki et al., 2002). Furthermore, it was found that deletion of the HogA MAP kinase, involved in osmoregulation, resulted in abnormal hyphae when grown on high-salt medium. Therefore, the behaviour of the *steC* mutant grown on media supplemented with different salt concentrations was studied. The *steC* mutant strain was not more severely inhibited by the salt than the wild type. Curled hyphae as in the *hogA* deletion strain were also not observed (results not shown). However, most of the conidiophores (70-80 %) displayed the altered morphology described above (Fig. 5.16). This phenotype was only observed in less than 10 % of wild type conidiophores.

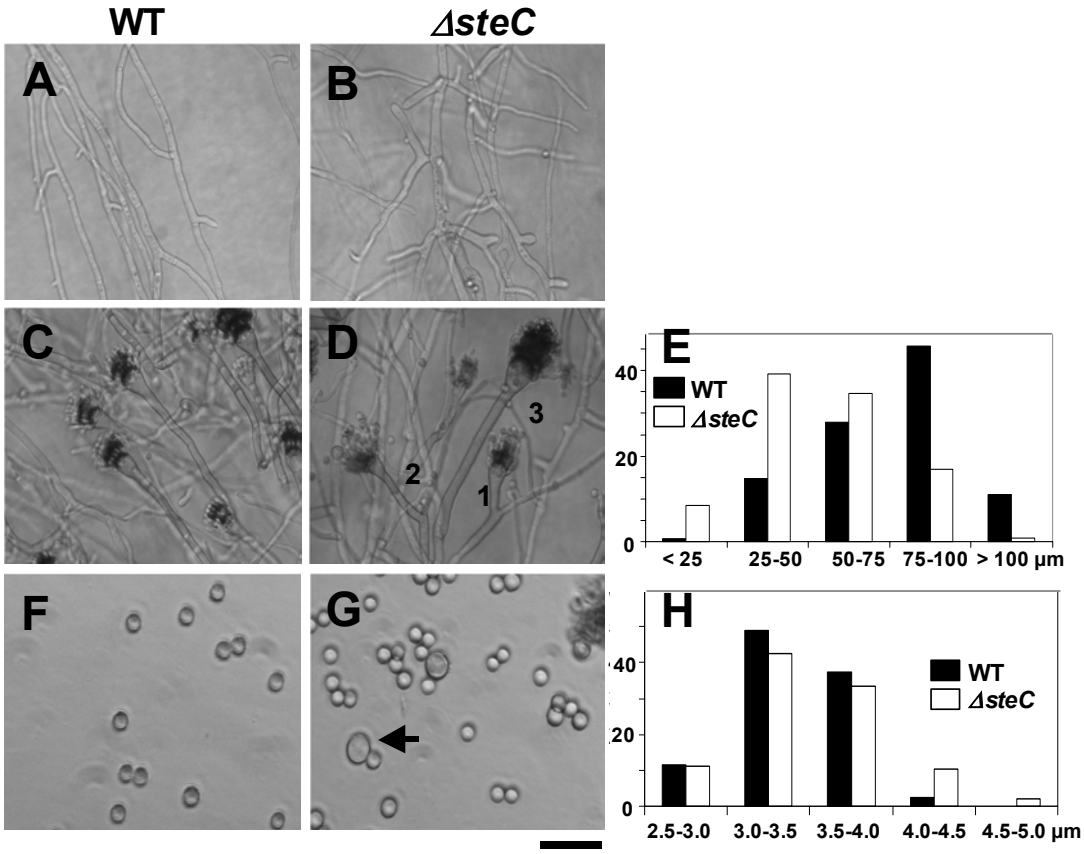

Fig. 5.16 Analysis of hyphae (A, B), conidiophores (C, D, E) and conidiospores (F, G, H) of a wild type (A, C, F) and a *steC* mutant (B, D, G). The numbers in (**D**) indicate three conidiophores of very different stalk-length. **(E)** The length of 200 conidiophores of wild type (black box) and *steC* mutant (white box) were measured and the values sorted into size categories. The numbers of conidiophores are given in %. The arrow in (**G**) points to an enlarged conidiospore. **(H)** The diameter of 300 spores of wild type (black box) and *steC* mutant (white box) were determined. The numbers of conidiospores are given in %.

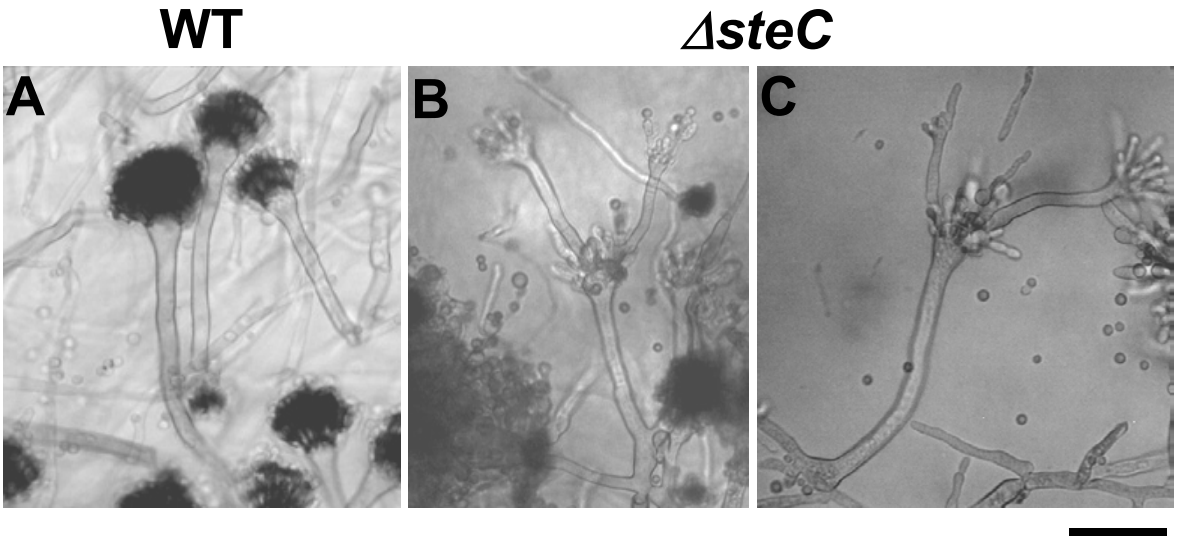

Fig. 5.17 Role of *steC* during conidiophore development. Phase contrast picture of conidiophores of a **(A)** wild type and a **(B, C)** *steC* mutant strain grown on minimal medium supplemented with 0.6 M KCl. The scale bar represents 25 µm.

5.2.4 Deletion of *steC* inhibits heterokaryon formation and sexual development

During the course of several times of crossing experiments, it became evident that *steC* mutant strains could not be crossed to other common laboratory *A. nidulans* strains (Fig. 5.18). Since the first step for a successful cross is the fusion of the hyphae, the question arose whether the failure of hyphae fusion or a later step was the cause for the observed sterility. To answer this question, protoplast fusion experiments were performed. The cell walls of a wild type (RMSO11) and a *steC* deletion strain (SWH51) were digested like for the preparation of a transformation, the washed protoplasts was mixed in osmotically stabilized medium, treated them with polyethylene glycol (PEG) to induce the fusion and plated them on minimal medium, on which neither the wild type nor the deletion strain would be able to grow, because of their nutritional requirements. Only heterokaryotic mycelium can grow. Colonies developed, which had the typical heterokaryotic appearance with conidiospores of both colors of the parent strains (Fig. 5.18). The same experiment was performed with two *steC* deletion strains (SWH51 and SWH33). Likewise, heterokaryotic mycelia was obtained. However, whereas the heterokaryon derived from a wild type and the *steC* deletion strain developed mature cleistothecia, the combination of two *steC* deletion strains proved to be sterile, although Hülle cells were produced. Further development of sexual structures, such as primordia or cleistothecia was never observed (Fig. 5.18).

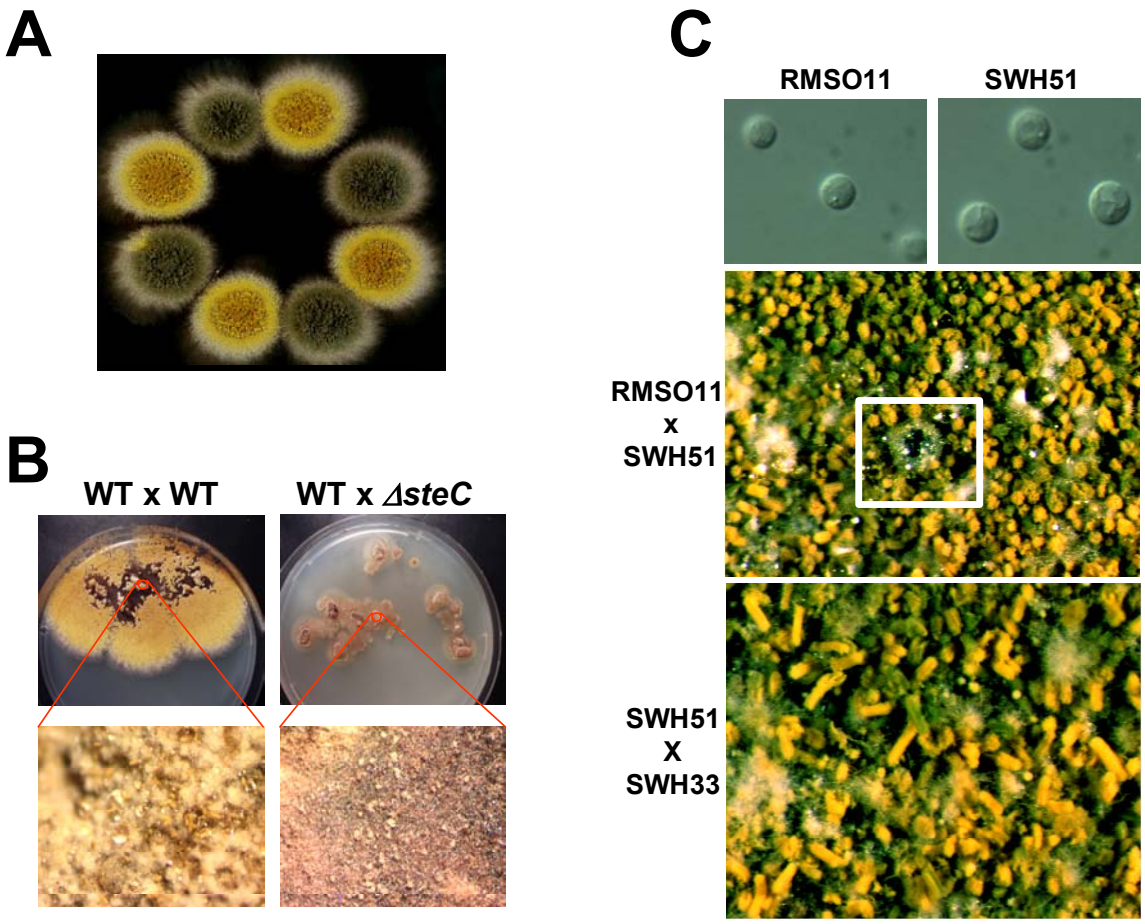

Fig. 5.18 Heterokaryon formation and sexual development. (**A**) Cross of two *A. nidulans* strains (green and yellow color). (**B**) After crossing, the agar pieces at the border of two different strains were transferred to a selective medium. Wild type (WT) crossed to a wild type resulted in heterocaryontic mycelium growing a selective medium, but wild type crossed to a *ΔsteC* mutant did not grow (**C**) Protoplasts were generated from a wild type (RMSO11) and two *ΔsteC* strains (SWH51 and SWH33) and used for protoplast fusion in different combinations. The figures show heterokaryon of the indicated strains. Green and yellow conidiophores are visible. The frame indicates a ripe, black cleistothecium. The heterokaryon of two *steC* deletion strains does produce green and yellow conidiophores but no cleistothecia.

5.2.5 *steC*-transcription is developmentally regulated and induced in metulae and phialides

The phenotypic characterization of the *steC* deletion strains suggested several important functions during the life cycle of *A. nidulans*. In order to answer whether the gene was transcriptionally regulated during development. RNA was isolated at different time points during

vegetative growth, asexual and sexual development and used for Northern blot analysis. It was found that the *steC* transcript was more abundant during conidiophore development. After asexual development was completed the signal decreased to a low level like in vegetative hyphae (Fig. 5.18).

In order to further specify the spatial expression of the gene, a C-terminal GFP-fusion construct was generated and introduced into a *steC* deletion strain. However, it did not complement the mutant phenotypes, suggesting that the GFP protein interferes with the catalytic function of SteC (results not shown). Fluorescence was detected only in the original transformation plates but not after re-streaking on minimal media plates. This suggests that the full-length fusion protein might be rather unstable. Because of this and the non-complementation of the mutant, another translational fusion of SteC and GFP was constructed, where GFP was fused before the catalytic domain (pHSRBgfp1) and introduced the plasmid into RMSO11. Intense GFP-fluorescence in metulae, phialides and young conidiospores and no fluorescence in conidiophore stalks was observed (Fig. 5.18). In older conidiophores, fluorescence was not detectable anymore suggesting that the increase of *steC* expression was only transient. This corresponds the results obtained in the transcript analysis described above. Promoter analysis revealed several putative binding sites for the developmental regulators StuA and AbaA (Andrianopoulos & Timberlake, 1994; Dutton et al., 1997). The fluorescence was not detectable during sexual development.

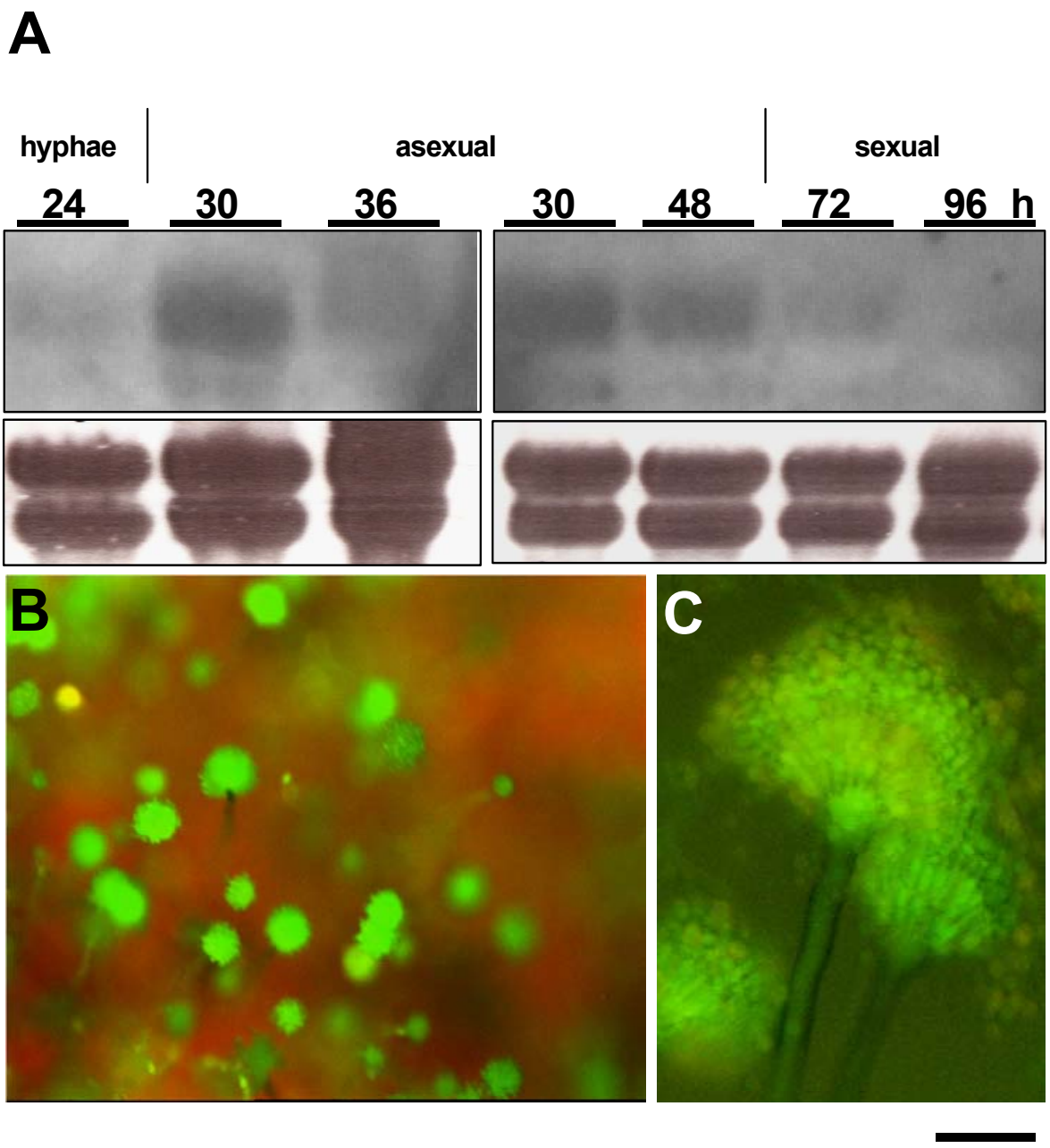

Fig. 5.18 Expression analysis of *steC*. **(A)** Northern blot analysis. RMSO11 was inoculated as a spore suspension on cellophane membranes on complete media and mycelium harvested after the time intervals indicated. 20 µg of total RNA was used for the Northern blot. A *steC*-specific fragment as a probe (^{32}P-labelled by PCR) was used. Before hybridisation the membrane was stained with methylene blue to visualize rRNA as a loading control (lower panels). **(B, C)** Expression of a translational GFP fusion construct in RMSO11 and detection of fluorescence in conidiophores observed on an agar plate. **(B)** Low-magnification overview where several conidiophore heads are visible. **(C)** Mature conidiophore. Metulae, phialides and young spores are brightly stained.

5.2.6 SteC activates at least two kinases

MAP kinase cascade signalling leads to the activation of MAP kinases by dual phosphorylation of a tripeptide motif TXY. The question raised which MAP kinases would be activated downstream of SteC during the life cycle of *A. nidulans*. For the detection of the phosphoepitopes monoclonal antibodies are commercially available. These antibodies recognize corresponding tripeptides in MAP kinases. Three different anti-phospho-antibodies (p44/42, SAPK/JNK and p38) were used to detect MAP kinases in *A. nidulans* under different physiological and developmental conditions. A wild type and a *steC* mutant strain for asexual development were induced, the mycelium harvested after different time points was used for the protein extracts in Western blot analyses. Signals were obtained with all three antibodies and one signal with the apparent molecular mass of 30 kDa detected with p44/42 was dependent on SteC (Fig. 5.19). A 42 kDa signal, detected with the p38 antibody, appeared to be increased upon developmental induction but was not dependent on SteC. Using the anti-phospho-SAPK/JNK antibody no reproducible change of the phosphorylation status was observed.

Since the SteC-dependent signal intensities obtained in the time-course experiment were rather low, it was further tried to detect the phosphorylation under different physiological conditions. In *S. cerevisiae* transient activation of MAP kinases can also be achieved after oxidative stress application. Therefore, it was tested whether some MAP kinases of *A. nidulans* were transiently phosphorylated under these conditions. Surprisingly, phosphorylation was detected not only after application of H_2O_2 but also by changing the medium. Under both conditions, and in agreement with the developmental phosphorylation pattern, phosphorylation of the kinase detected with p44/42 was SteC-dependent. In addition, the phosphorylation of a kinase (49 kDa) detected with the anti-phospho-SAPK/JNK antibody was stimulated by SteC (Fig. 5.19).

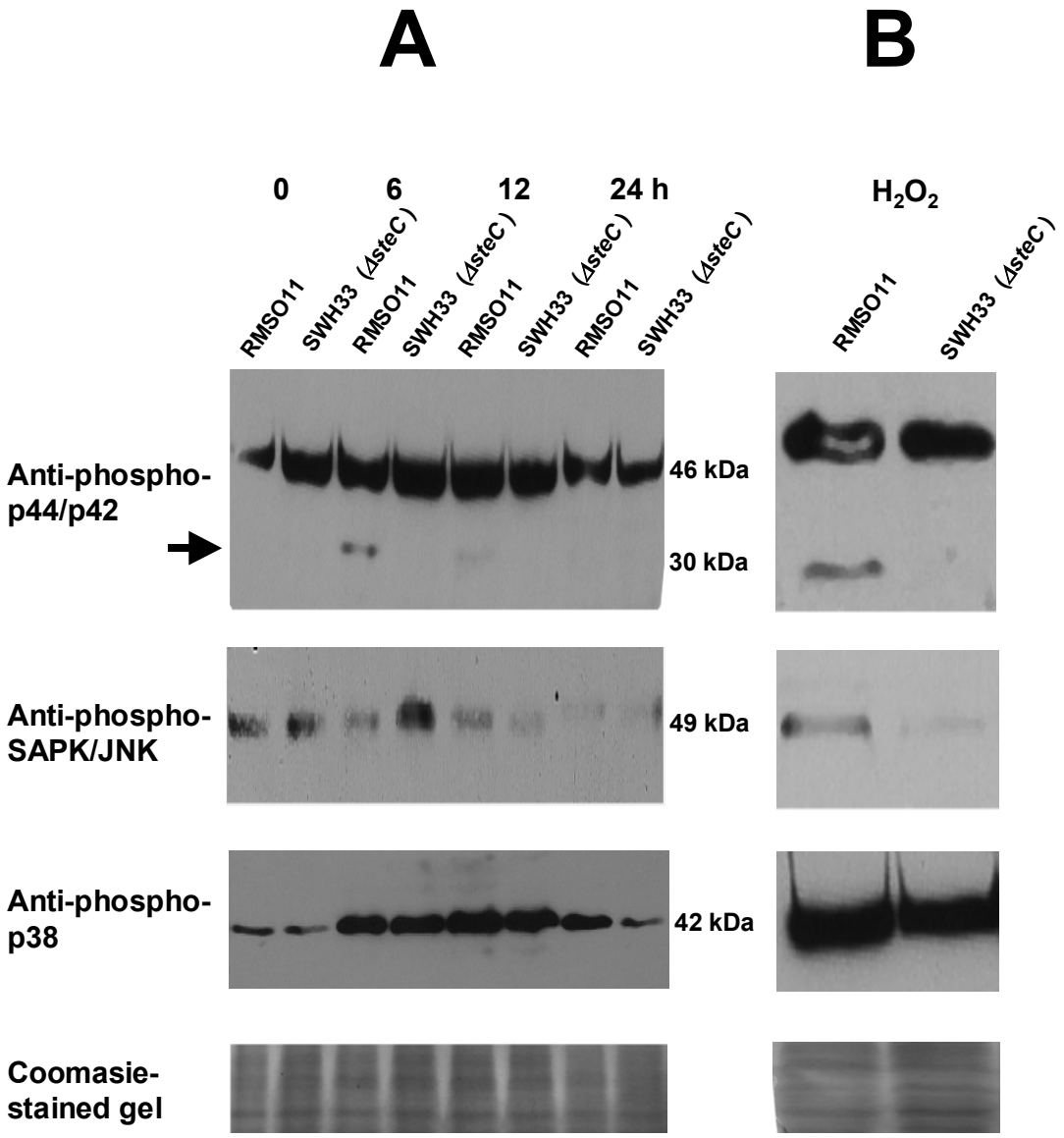

Fig. 5.19 SteC-dependent phosphorylation of MAP kinases. (A) Time course of asexual development. Protein extracts (RMSO11 and SWH33) were subjected to a western blot analysis with the antibodies indicated. The arrows in the first row of pictures point to the MAP kinase, which is SteC-dependent phosphorylated. **(B)** Induction of the phosphorylation by oxidative stress or change of the media (control). As a loading control the same amounts of protein were separated as used for the western blots on a SDS gel and stained it with Coomasie. The pictures are shown below the western blots. For further details see the methods section.

6. Discussion

The fungus *A. nidulans* has two distinctive reproductive developmental processes: asexual and sexual developments. Several genes involved in asexual development have been genetically characterized and the interactions between them have been investigated (for a review, see (Adams et al., 1998; Fischer, 2002). In contrast, the sexual developmental process has not been well dissected. The initial step of sexual development is the aggregation of vegetative mycelia. Some cells in the aggregates differentiate to Hülle cells and some to primodia. Cleistothecia are later developed from primodia. In a cleistothecium, asci are formed in which eight ascospores are produced by meiosis (for a review, see Zonneveld, 1977). Although some genes related to sexual development have been successfully identified including *veA* (Champe et al., 1981; Kim et al., 2002), *tubB* (Kirk & Morris, 1991), *medA* (Busby et al., 1996), *stuA* (Wu & Miller, 1997), *nsdD* (Han et al., 2001) and *steA* (Vallim et al., 2000), *lsdA* (Lee et al., 2001), *phoA* (Bussink & Osmani, 1998), *dopA* (Pascon & Miller, 2000), *sakA* (*hogA*)(Kawasaki et al., 2002; Han & Prade, 2002), the molecular genetic mechanisms controlling sexual reproduction of *A. nidulans* are still unclear.

In this study mainly three genes *mutA, hgtA* and *steC* related to the process of sexual development in *A. nidulans* were studied.

6.1 The carbon cycle during the sexual development in *A. nidulans*

6.1.1 MutA is expressed during sexual development in *A. nidulans* and mobilizes mutan

For many organisms, carbohydrates are important carbon and energy sources. In earlier publications Zonneveld (1973) found that α-1,3 glucan (mutan) accumulates in *A. nidulans* during vegetative growth and becomes metabolised during sexual development. He could show that the presence of 2-deoxy-D-glucose (2-DG) inhibits the synthesis of mutan and sexual development did not occur although there was no severe effect on vegetative growth. He concluded that sexual development strictly depends on the formation and subsequent degradation of mutan. In the later literature, mutan was considered as one of determined factors in cleitothecium formation (Braus et al., 2002). In our molecular approach, surprisingly, I found that the *mutA* gene is not essential for cleistothecium formation. Although it cannot be ruled out that

in the absence of the MutA enzyme mutan is partly degraded by some other enzymes, it is found that the degradation is greatly affected. Therefore it appears to be unlikely, that *A. nidulans* possesses several enzymes with α-1,3-glucanase activity. A second reason why *mutA* deletion strains are still able to form fruiting bodies could be that other carbon sources are consumed during sexual development. Likewise, *A. nidulans* accumulates a variety of carbohydrate polymers besides mutan during vegetative growth (Zonneveld, 1973). Recently, it was shown that at the onset of sexual development many different cell wall lytic enzymes are produced and thus many of the polymers are probably degraded (Prade et al., 2001). The degradation of these alternative polymers could be also inhibited by 2-deoxy-D-glucose. Alternatively, the presence of 2-DG could also interfere with the pathways involved in sexual development. This would explain why in earlier experiments sexual development was blocked in the presence of 2-deoxy-D-glucose (Zonneveld, 1973).

Mutanase (α-1,3-glucanase) has been characterised in *P. purpurogenum* and *T. harzianum*. Here it was found to be induced in the presence of mutan isolated from *Streptococcus mutans* cultures (Fuglsang et al., 2000). The authors proposed a mutan binding domain in the C-terminal of two mutanases of *Penicillium* and *Trichoderma*. However, this domain was not found in the mutanase of *A. nidulans.* This might be due to the different roles of the enzymes in the different organisms. In *Penicillium* and *Trichoderma* the enzyme is secreted to degrade extracellular mutan. In contrast, it is believed that *A. nidulans* secretes the enzyme into the cell wall, where it mobilizes the polymer. Since the mutan in the cell wall might be structurally different from insoluble mutan it could be that the binding capability of MutA of *A. nidulans* does not rely on the domain postulated in *Penicillium*. It could also be that the binding of *A. nidulans* MutA requires another component present in the cell wall but not in *Streptococcus* mutan.

Compared with wild type, *mutA* deletion and overexpression strains did not show evident differences with regards to vegetative growth, asexual development or sexual fruiting body formation, the number of cleistothecia per cm^2, the number of ascospores per cleistothecium, the viability of the ascospores in normal minimal and complete media. But, the amount of "alkali-soluble fraction" (mutan) in the wild type after 12 days of growth was always about 50 % of the mutan in the *mutA* mutant, the amount of mutan in overexpression strain was slightly lower than in wild type. This suggested that MutA indeed functions in mutan degradation. If approximately estimating the mutan fraction as the amount of "alkali-soluble fraction" of a *ΔmutA* strain minused by that of a *mutA* overexpression strain after longer growth, the mutan is just around 5-

8% of the amount of supplemented carbon source such as glucose in normal minimal and complete media. This explained why no evident phenotypes between *ΔmutA* and wild type and *mutA* overexpression strains were observed in normal media supplemented with enough carbon source, but evident phenotypes were observed in the medium just using the mutan fraction as carbon source. In addition, MutA was specifically expressed in Hülle cells. This suggested mutan was reutilized in the stage of sexual development, but appeared to play a minor role in the contribution to fruiting body development. The higher density growth of overexpression and wild type strain in the medium using mutan as carbon source, together with the signal peptide in the N-terminus of MutA, suggested MutA is secreted to utilize the mutan. The *gpd::mutA* constitutive overexpression strains of MutA did not show lysis of the mycelium. In addition, MutA expression in wild type during sexual development did not show mycelium autolysis. This suggests that the components of cell wall change dynamically during the life cycle of *A. nidulans* and that mutan is probably not a fundemental skeletal component in *A. nidulans* cell wall.

Transcript and *sgfp* reporter construct analysis showed that the expression of *mutA* is highly regulated and specifically induced in Hülle cells and some connecting hyphae. Thus, this gene opens the possibility to study the regulatory elements as well as regulators. Three DNA-binding protein regions of the putative regulatory factors were identified. A new method was used for DNA-binding protein isolation. In the preliminary experiments, it was found that a 40 kDa protein bound specifically to a 150 bp fragment at the –1.7 kb position of *mutA* promoter. This protein needs to be identified using Mass-spectrum technology in the future study. Further studies of regulatory elements which specifically bind will be of great importance in the analysis of the transcription regulation mechanism of the *mutA* gene and other sexual pathway specific genes.

The experiments suggested another interesting aspect of the nutrition of a developing mycelium. The consumption of cell wall polymers and the production of cell wall lytic enzymes is a critical process. However, it was found that the expression of the mutanase gene in *A. nidulans* is restricted to a certain type of cell, namely the Hülle cells. The cell walls of these cells do not contain mutan (Zonneveld, 1977). Therefore it is likely that Hülle cells produce mutanase, that other substrate hyphae are partly degraded and that the Hülle cells or the connecting hyphae absorb the released carbohydrates. Hence, Hülle cells could provide the nutrition to the cleistothecium and thus they could have a nurse cell function. This raises of course the question about the uptake of the released hexose and how it is provided to the developing cleitothecium.

To address this question, in the present study, a putative high-affinity hexose transporeter, found in the SSH library (Scherer, 2001), was characterized.

6.1.2 *hgtA* encodes a high affinity glucose transporter and is expressed in ascogenous hyphae

Bacteria and fungi can secrete some enzymes that degrade long chain carbohydrate polymers from plant debris in the soil. The degradation leads to the release of hexose and other monosaccharide. In order to reutilize those monosaccharides, it is necessary to transfer them over the cell membrane by a group of hexose transporter proteins. In this work, a high-affinity hexose transporter gene from *A. nidulans* was molecularly studied. *hgtA* encodes a 59 kDa protein with substantial similiarity to hexose transporeters from other fungi. The hydrophobicity prediction showed the presence of 12 putative transmembrane (TM) domains, a characteristic feature of the major facilitator superfamily (Marger & Saier, 1993).

The deletion strain of *hgtA* didn't show evident phenotypes. However, this might not be surprisingly, given that at least 10 different hexose transporters were found in the *A. nidulans* partial genome sequence (Weber, 2002). Therefore, it is likely that other transporters are able to substitute the function of *hgtA*. In *S. cerevisiae*, HXT1-4 and HXT6-7 encode the major hexose transporters. The expression of each of those genes in the MC996 background strain deleted for HXT1-7 allows growth on glucose (Reifenberger et al., 1995). Likewise, in the yeast *K. lactis*, two glucose transporters, the high-affinity transporter Hgt1 and the low-affinity transporter Rag1, have been found (Wesolowski-Louvel et al., 1992; Billard et al., 1996). Although, no other Rag1 and Hgt1-related sequences have been found in the *K. lactis* genome, the deletion of both genes did not completely prevent glucose transport, indicating that there are even more and probably unrelated glucose transporters in *K. lactis*. The same might also be the case on *P. stipitis* (Weierstall et al., 1999).

The *hgtA* gene was isolated as a gene expressed during sexual development and *sgfp* reporter constructs revealed the expression in the cells inside the cleitothecium. Most likely, these cells are the ascogenous hyphae. In contrast, Hülle cells showed no expression. In the shell of a cleitothecium, a shining fluorescence was also be seen. This result suggested the carbon supply in ascospore maturation and cleitothecium development occur through HgtA to transport the hexose from the medium or MutA-released glucose (Fig. 6.1). It will be the challenge of the future

research to unravel the exact nature of the carbon flow during the stage of sexual development of *A. nidulans.*

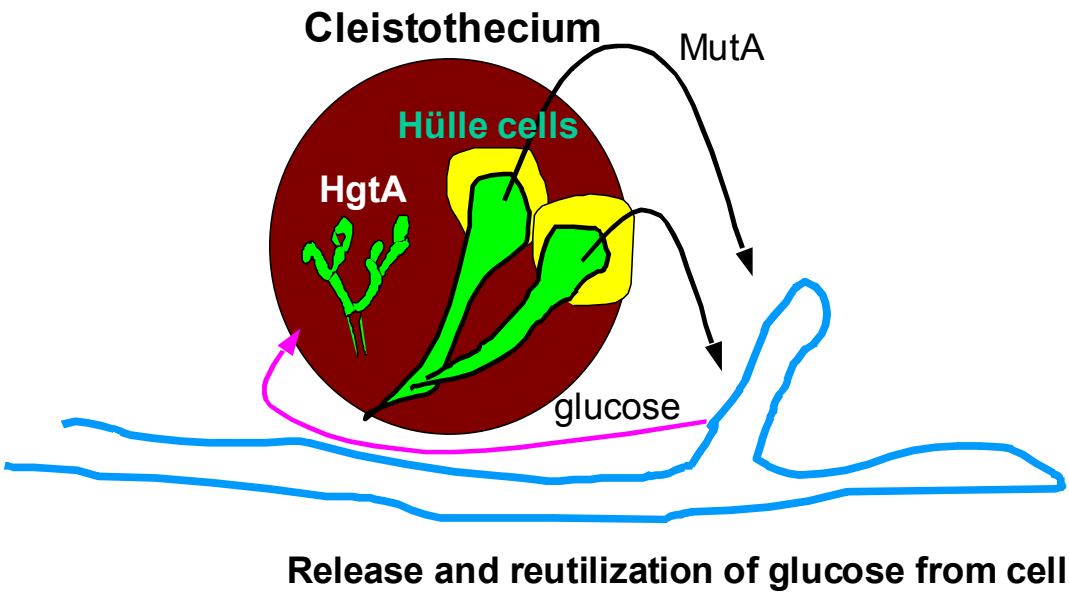

Fig. 6.1 Model of MutA and HgtA functions. MutA is expressed in Hülle cells and secreted for degrading mutan in hyphae. HgtA transports the released glucose into the cleistothecium for supplying the carbon and energy source for the formation and maturation of the cleistothecium.

6.2 The MAPKK-kinase SteC regulates conidiophore morphology and is essential for heterokaryon formation and sexual development in *A. nidulans*

In addition to search for the target genes that are involved in sexual development of *A. nidulans.* The identification of the upstream factors, possibly involved in the regulation of the expression of the target genes, were attempted. In this work I found that SteC and thus a MAP kinase cascade is required for hyphal extension, conidiophore morphogenesis, heterokaryon, and cleistothecium formation. Many of the observed phenotypes are similar to the ones described for a corresponding MAPKK kinase mutant, *nrc-1,* of *N. crassa* (Kothe & Free, 1998). This related fungus undergoes two developmental pathways, an asexual and a sexual one. Asexual spore formation occurs after nutrient depletion. *nrc-1* mutants grow slower, display a fertilization defect and induce asexual development under non-starvation conditions (*nrc* = *non-repressed conidiation*). In addition, the spores appear to have a developmental block and do not separate

easily. The appearance of the chains of spores resembles the phenotype of *abaA* mutants of *A. nidulans*, where phialides do not mature to produce conidia but elongate and swell in regular intervals (Andrianopoulos & Timberlake, 1994). Despite the phenotypic similarities between the *nrc-1* mutant of *N. crassa* and the *steC* mutant of *A. nidulans,* a comparison to the results in *S. cerevisiae* is more elusive to speculate about actions and interactions of SteC in *A. nidulans.*

6.2.1 Hyphal extension, conidiophore and conidial development

Deletion of *steC* impaired hyphal extension. Although it is not obvious which signals might trigger hyphal elongation and might be transmitted by a MAP kinase cascade, there is more evidence that phosphorylation events are important for this process. It has been shown that a MAP kinase (MpkA) is involved in hyphal growth and thus SteC could be involved in the regulation of the activity of this kinase although it is no experimental evidence for that (see below) and the *mpkA* is much more severe than the *steC* mutation (Bussink & Osmani, 1999). In addition to MpkA, several other kinases have been shown to be required for efficient cell wall extension and SteC thus could also be upstream of those kinases (Chen et al., 2000; Navarro-Garcia et al., 1998; Yarden et al., 1992).

Another effect of the *steC* mutation, is the disturbance of normal conidiophore and conidiospore development. The height of the stalks and the diameter of the spores showed a difference to wild type. This phenotype resembled the phenotype of the *A. nidulans dopA* mutant (Castiglioni Pascon & Miller, 2000). The *dopA* encodes a leucin-zipper transcription factor. The *dopA* mutant strains fail in addition to initiate cleistothecium formation, which also is comparable to *steC* mutants. However, further experiments are needed for a detailed study of a possible relationship between SteC and DopA. In addition to the spore and conidiophore stalk defects in *steC* mutants, metulae do not differentiate properly and produce phialides, but instead re-differentiate into hyphae or conidiophore stalks, resulting in secondary condiophores. Here, comparison to *S. cerevisiae* could help to explain the role of a MAP kinase cascade. Metula differentiation and phialide production resembles pseudohyphal development in yeast and homologous regulators appear to be involved in the regulation of the two processes (Gavrias *et al.*, 1996; Mösch, 2002). Since this differentiation depends on a MAP kinase cascade in *S. cerevisiae*, it is also likely to be the case in *A. nidulans* (Gustin et al., 1998). The MAP kinase responsible for the final transmittance of the signal to a transcription factor could be the 30 kDa

protein, which was identified. In *S. cerevisiae* two MAP kinase substrate transcription factors Tec1 and Ste12 are involved in pseudohyphal growth regulation (Mösch, 2002). In *A. nidulans* the homologue of Tec1 is AbaA, a transcription factor, which is involved in phialide differentiation. Hence, this factor would be one good candidate for a target of the MAP kinase cascade (Andrianopoulos & Timberlake, 1994). Although, the phenotypes of *steC* and *abaA* null mutants are different, it could be that modulation of the AbaA activity in *steC* deletion strains results in a different phenotype than deletion of the entire *abaA* gene. Similar phenomena were reported for another important regulator of conidiophore development, BrlA (Prade & Timberlake, 1993). In addition to the Tec1 homologue AbaA, a transcription factor with similarity to Ste12 was also described in *A. nidulans*, but deletion of the gene had no obvious phenotype on conidiophore development (Vallim, et al., 2000). Additional experiments will be required to unravel the nature of interactions between those regulators and the MAP kinase cascade in *A. nidulans.*

Since the defective phenotype of the *steC* mutant is found in a small percentage of conidiophores, other cascades likely can substitute for the function of SteC. However, we observed that under high osmolarity conditions the phenotype was much more severe. This might be explained as follows: The transcription of the *hogA* gene is induced during conidiophore formation and thus the protein amount is likely to be high during asexual development. High osmotic pressure leads to an activation of HogA (= SakA) and this might interact with the developmental program triggered by the 30 kDa MAP kinase (Han & Prade, 2002; Kawasaki et al., 2002) Phosphorylated HogA could negatively influence the SteC pathway especially when SteC is absent. Similarly, Hog1 appears to be involved in the regulation of morphogenesis in *C. albicans* (Alonso-Monge et al., 1999). Such cross-talk between different MAP kinase pathways is typical for these signaling modules and the analysis of the nature of its regulation is one important field of research (Madhani & Fink, 1998; Madhani et al., 1997; Sabbagh et al., 2001). Similarly, Kawasaki et al. (2002) suggested cross talk to explain precocious formation of sexual structures in *sakA* mutants.

Despite an obvious effect of osmolarity on conidiophore development in *steC* mutants, we did not observe an increased sensitivity of hyphal growth against high osmolarity, comparable to the phenotype observed when the corresponding MAP kinase, HogA, is not functional (Han & Prade, 2002). This is not surprising because alternative, SteC-independent pathways might exist. Likewise, in *S. cerevisiae* three pathways were described (O'Rourke & Herskowitz, 2002).

6.2.2 Heterokaryon and cleistothecium formation

Heterokaryon formation could be compared to the mating reaction in *S. cerevisiae*. The latter involves a pheromone-receptor system through which yeast cells of opposite mating type recognize each other (Banuett, 1998). However, *A. nidulans* is a homothallic fungus and does not require a mating partner of opposite mating type for a successful mating reaction (Coppin et al., 1997). Nevertheless, there is mounting evidence that homothallic fungi harbour and express mating type genes in the same hyphae and thus mimic the presence of a compatible partner. In *Sordaria macrospora* it was shown that homologues of ascomycetous mating type genes exist, and that they can complement corresponding mutants of the heterothallic species *Podospora anserina* (Pöggeler & Kück, 2001). However, genes encoding a peptide-pheromone like in *S. cerevisiae* have not yet been detected. Besides peptide-pheromones other low molecular weight compounds could be involved in the signalling between hyphae. In *A. nidulans* a system of interconvertible fatty acid derivatives, the PSI factors, have been described, which trigger developmental decisions (Champe & El-Zayat, 1989; Champe et al., 1987). Application of one of the compounds leads to a precocious induction of the sexual cycle. In addition, the reaction requires a high density of cells, suggesting another sensing mechanism. The question about the signal recognized by hyphae is certainly one of the most interesting ones to be solved in the future. The fact that *steC* mutants of *A. nidulans* could not be crossed to other laboratory strains with intact *steC* alleles could involve in such a communication system. Both partners have to recognize each other and prepare the hyphae to fuse. This probably requires local lysis and remodeling of the cell walls and subsequent fusion and resealing. The induction of the corresponding genes or the triggering of the corresponding enzyme activities could be the role of the MAP kinase cascade.

Another phenomenon, which might be explained with similar arguments, is the lack of cleistothecium formation in *steC* mutant strains. These strains are still able to form Hülle cells, but fail to continue with the development to produce mature cleistothecia. Since mating types are also required for nuclear recognition in the ascus mother cell and for nuclear fusion, it could well be that this process is inhibited in *steC* mutants (Coppin & Debuchy, 2000; Debuchy, 1999). Whether the signals transmitted in this process in *A. nidulans* are the same as for heterokaryon formation is unknown. In *S. cerevisiae* the downstream transcription factor for mating is Ste12.

Similarly, it could be that a homologue of this protein is involved in mating and fruiting body formation in *A. nidulans*. Recently, a gene encoding a protein with similarity to Ste12 was characterized and indeed, deletion strains were sterile but produced masses of Hülle cells. The mutation was recessive (Vallim, et al., 2000). However, the phenotype is distinct from the one of *steC* deletion strains.

6.2.3 Identification of SteC targets

After discussing the different signals and environmental conditions, which might be SteC-dependent transmitted, the question of the MAP kinase(s) downstream of SteC will be addressed. Using antibodies against conserved phosphoepitopes, I detected several kinases and showed that at least two were phosphorylated in a SteC-dependent manner. It was tried to detect phosphorylation of specific proteins after heterokaryon formation, during asexual and during sexual development. However, these processes are likely to be too slow in order to detect changes of the phosphorylation status. In *S. cerevisiae* phosphorylation of Fus3 occurs within 5 minutes after pheromone addition and decreases steadily thereafter (Sabbagh, et al., 2001). In comparison, conidiophore development requires several hours and sexual differentiation even several days. In addition, cultures are not well enough synchronized to detect such transient phosphorylation events. However, after stress activation I found two kinases dependent on SteC. The sequences of four different MAP kinases have been deposited in the databases, HogA (SakA) (Acc. no. AF270498)(Han & Prade, 2002), MpkA (Acc. no. U59214)(Bussink & Osmani, 1999), MpkB (Acc. no. 198118) and MpkC (Acc. no. AF195773). I detected one MAP kinase, which was recognized by the anti-phospho antibody p42/p44. This antibody recognizes the tripeptide TEY, which is found in MpkA and MpkB. The predicted molecular masses of these two kinases are 47.8 and 41.6 kDa, respectively. However, the SteC-dependent MAP kinase which is detected in my experiments and which was phosphorylated during asexual development had an apparent molecular mass of 30 kDa. This suggests that a new MAP kinase is involved in the regulation of development. Indeed, MpkA is required for spore germination and hyphal growth (Bussink & Osmani, 1999) and MpkB has not been functionally characterized yet although there is some evidence for an involvement in sexual development (Jahng, Chonbuk, University of South Korea, personal communication). The antibody SAPK/JNK recognizes the tripeptide TPY, which is not found in any known *A. nidulans* MAP kinase. The p38 antibody detects the tripeptide TGY in a

protein with an apparent molecular mass of 42 kDa. This peptide motif is found in MpkC and HogA (SakA). The predicted molecular masses of MpkC and HogA are 39.5 and 43.2 kDa, respectively. Thus one of them could be the one detected in our experiments. Although this protein was induced upon development, its phosphorylation was not dependent on SteC. If it were HogA, this result would not be surprising since three pathways for osmosensing exist in *S. cerevisiae*, two of which are independent on Ste11 (Furukawa et al., 2002). Similarly, Kawasaki et al. (2002) found transient activation of SakA upon induction of asexual development. However, the time periods where the phosphorylated species of SakA was detectable varied in my experiment from the reported data. This discrepancy may be explained by slightly different experimental conditions. As it have been seen phosphorylation of SakA occurs very rapidly after application of stress or changing of medium.

The *steC* mutant strain (SWH51) was also respectively transformed by several transcription factors and target genes. A fluffy phenotype appeared similar in the transformants of *steC* mutant and wild type strains after transformation by a *rosA* overexpression construct (Vienken, 2003), or a *fadA* dominant mutant construct (Hicks et al., 1997). The bright fluresence also appeared in the Hülle cells after the transformation of SWH51 by the construct of *mutA* promoter fused with *sgfp* (Wei et al., 2001)(results not shown). Those results suggested RosA, FadA and MutA act in different pathways with SteC. The identification of the SteC targets, as well as the discovery of other unknown MAP kinases involved in the morphological transitions, will be one of the goals for future research.

6.3 Outlook

The regulatory pathway or network of the genes that are involved in the asexual reproduction process, from signaling of differentiation to completion of conidiation, is now well established (Adams, et al., 1998). The signal transduction pathways that include *fluG, fadA* and *flbA* have been shown to establish a balance between filamentous growth and development through regulation of *brlA* expression (Adams, et al., 1998). Early *brlAβ* expression initiates development by activateing *abaA* expression. A positive feedback loop is established in which *abaA* enhances *brlAα* expression and guarantees commitment to terminal differentiation (Mirabito et al., 1989; Han et al., 1993; Prade & Timberlake, 1993). Correct spatiotemporal expression of both *brlA* and *abaA* are required for asexual differentiation (Miller et al., 1992; Aguirre, 1993). Furthermore,

the balance between *brlAα* and *brlAβ* expression is particularly important throughout development for correct conidiophore morphogenesis and conidial differentiation (Prade & Timberlake, 1993; Busby, et al., 1996).

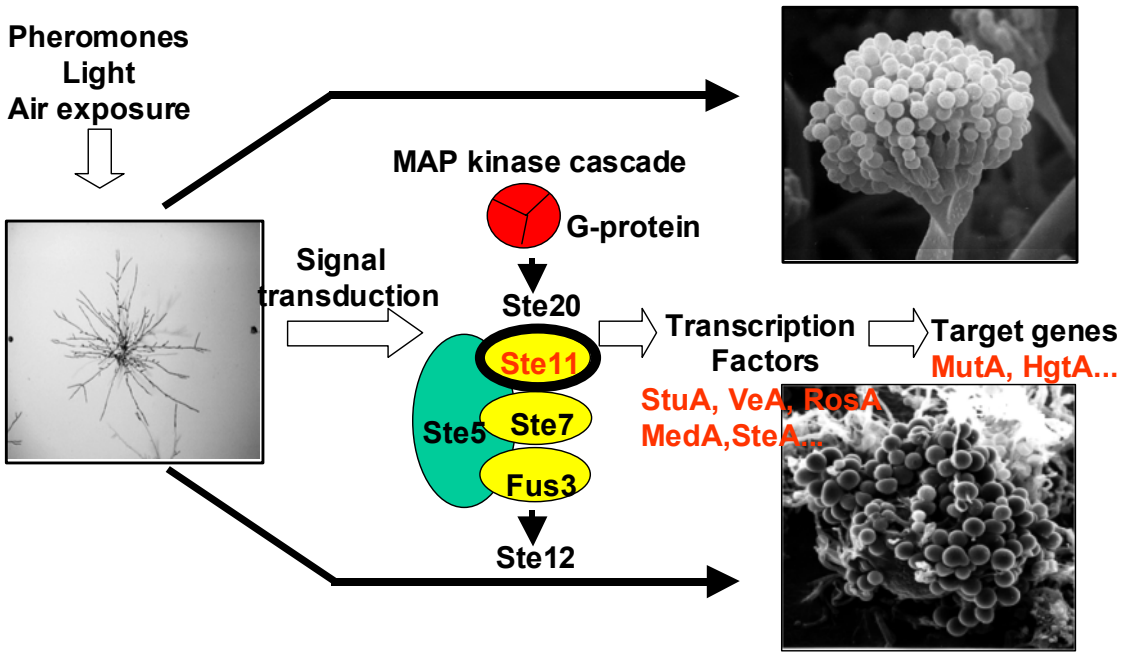

Fig. 6.2 Scheme of the regulatory pathway in sexual development of *A. nidulans* and the positions of genes studied here (MutA, HgtA and SteC). The signals that initiate sexual development in *A. nidulans* are transferred by signal transduction pathways to some transcription factors that control the expression of target genes. A MAPK cascade from *S.cerevisiae* is used as a putative model of the signal transduction in the sexual development of *A. nidulans*.

However, information about the sexual cycle regulatory network and potential SteC target genes is limited. Potential signaling molecules such as Aras, heteromeric Gα and Gβ proteins and RGS protein and a few developmental-specific components in MAP kinase cascade such as MpkA (Bussink & Osmani, 1999), SteA (Vallim, et al., 2000) and HogA(=SakA) (Han & Prade, 2002; Kawasaki, et al., 2002) have been reported in *A. nidulans*. However, a complete signaling pathway has not yet been described. Like other higher eukaryotes, *A. nidulans* uses multiple-signal inputs to make developmental decisions leading to organogenesis and the formation of multicellar structures. The *A. nidulans* life cycle requires the coordination of mitotic and meiotic reproductive cycles. Regulatory functions of BrlA and AbaA are restricted to the asexual developmental programme. By contrast, MedA, StuA and SteC are required during both

reproductive developmental cycle (Vallim, et al., 2000; Wei et al., 2003). In recent years, the number of reports about research in sexual development of *A. nidulans* has increased. Those genes include genes involved in signal transduction, transcription factors and some target genes. With the increasing number of genes involved in sexual development, a clear network of regulaters is likely to emerge in the near future (Fig. 6.2).

7 Literature

Adams, T. H., Hide, W. A., Yager, L. N. & Lee, B. N. (1992). Isolation of a gene required for programmed initiation of development by *Aspergillus nidulans*. *Mol. Cell. Biol.* **12**, 3827-3833.

Adams, T. H., Wieser, J. K. & Yu, J.-H. (1998). Asexual sporulation in *Aspergillus nidulans*. *Microbiol. Mol. Biol. Rev.* **62**, 35-54.

Aguirre, J. (1993). Spatial and temporal controls of the *Aspergillus brlA* developmental regulatory gene. *Mol. Microbiol.* **8**, 211-218.

Alonso-Monge, R., Navarro-Garcia, F., Molero, G., Diez-Orejas, R., Gustin, M., Pla, J., Sanchez, M. & Nombela, C. (1999). Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J. Bacteriol.* **181**, 3058-3068.

Andrianopoulos, A. & Timberlake, W. E. (1994). The *Aspergillus nidulans abaA* gene encodes a transcriptional activator that acts as a genetic switch to control development. *Mol. Cell. Biol.* **14**, 2503-2515.

Aramayo, R., Adams, T.H. & Timberlake, W.E. (1989). A large cluster of highly expressed genes is dispensable for growth and development in *Aspergillus nidulans. Genetics.* **122,** 65-71.

Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1995). *Short protocols in molecular biology*: John Wiley & Sons, Inc.

Banuett, F. (1998). Signalling in the yeasts: an informational cascade with links to the filamentous fungi. *Microbiol. Mol. Biol. Rev.* **62**, 249-274.

Billard P., Menart S., Blaisonneau J., Bolotin-Fukuhara M., Fukuhara H. & M., W.-L. (1996). Glucose uptake in Kluyveromyces lactis: role of the HGT1 gene in glucose transport. *J Bacteriol* **178**, 5860-5866.

Bisson, L. F., Coons, D. M., Kruckeberg, A. L. & Lewis, D. A. (1993). Yeast suger transporters. *Crit. Rev. Biochem. Mol. Biol* **28**, 259-308.

Boles E. & Hollenberg, C. P. (1997). The molecular genetics of hexose transport in yeasts. *FEMS Microbiol. Rev.* **21**, 85-111.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**, 248-254.

Braus, G. H., Krappmann, S. & Eckert, S. E. (2002). Sexual development in ascomycetes - Fruit body formation of *Aspergillus nidulans*. In *Molecular biology of fungal development*, pp. 215-244. Edited by H. D. Osiewacz. New York: Marcel Decker.

Busby, T. M., Miller, K. Y. & Miller, B. L. (1996). Suppression and enhancement of the *Aspergillus nidulans medusa* mutation by altered dosage of the *bristle* and *stunted* genes. *Genetics* **143**, 155-163.

Bussink, H. J. & Osmani, S. A. (1998). A cyclin-dependent kinase family member (PHOA) is required to link developmental fate to environmental conditions in *Aspergillus nidulans*. *EMBO J.* **17**, 3990-4003.

Bussink, H. J. & Osmani, S. A. (1999). A mitogen-activvated protein kinase (MPKA) is involved in polarized growth in the filamentous fungus *Aspergillus nidulans*. *FEMS Microbiol. Lett.* **173**, 117-125.

Casselton, L. & Zolan, M. (2002). The art and design of genetic screens: filamentous fungi. *Nature Reviews Genetics* **3**, 683-697.

Castiglioni Pascon, R. & Miller, B. L. (2000). Morphogenesis in *Aspergillus nidulans* requires dopey (DopA), a member of a novel family of leucine zipper-like proteins conserved from yeast to humans. *Mol. Microbiol.* **36**, 1250-1264.

Chaleff, D. T. & Tatchell, K. (1985). Molecular cloning and characterization of the *STE7* and *STE11* genes of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **5**, 1878-1886.

Champe, S. P. & El-Zayat, A. E. (1989). Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. *J. Bacteriol.* **171**, 3982-3988.

Champe, S. P., Kurtz, M. B., Yager, L. N., Butnick, N. J. & Axelrod, D. E. (1981). Spore formation in *Aspergillus nidulans*: Competence and other developmental processes. 255-276.

Champe, S. P., Nagle, D. L. & Yager, L. N. (1994). Sexual sporulation. In *Prog Ind Microbiol*, pp. 429-454. Edited by S. D. Martinelli & J. R. Klinghorn. Amsterdam: Elsevier.

Champe, S. P., Rao, P. & Chang, A. (1987). An endogenous inducer of sexual development in *Aspergillus nidulans*. *J. Gen. Microbiol.* **133**, 1383-1387.

Chen, J., Zhou, S., Wang, Q., Chen, X., Pan, T. & Liu, H. (2000). Crk1, a novel Cdc2-related protein kinase, is required for hyphal development and virulence in *Candida albicans*. *Mol. Cell. Biol.* **20**, 8696-8708.

Clutterbuck, A. J. (1969). A mutational analysis of conidial development in *Aspergillus nidulans*. *Genetics* **63**, 317-327.

Clutterbuck, A. J. (1992). *Aspergillus: Biology and Industrial Applications*. Boston, Massachusetts: Butterworth-Heinemann.

Coppin, E. & Debuchy, R. (2000). Co-expression of the mating-type genes involved in internuclear recognition is lethal in *Podospora anserina*. *Genetics* **155**.

Coppin, E., Debuchy, R., Arnaise, S. & Picard, M. (1997). Mating types and sexual development in filamentous ascomycetes. *Microbiol. Mol. Biol. Rev.* **61**, 411-428.

Debuchy, R. (1999). Internuclear recognition: A possible connection between euascomycetes and homobasidiomycetes. *Fungal Genet. Biol.* **27**, 218-223.

Dutton, J. R., Johns, S. & Miller, B. L. (1997). StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. *EMBO J.* **16**, 5710-5721.

Fischer, R. (2002). Conidiation in *Aspergillus nidulans*. In *Molecular biology of fungal development*, pp. 59-86. Edited by H. D. Osiewacz. New York: Marcel Decker.

Fuglsang, C. C., Berka, R. M., Wahleithner, J. A., Kauppinen, S., Shuster, J. R., Rasmussen, G., Halkier, T., Dalboge, H. & Henrissat, B. (2000). Biochemical analysis of recombinant fungal mutanases. *J. Biol. Chem.* **275**, 2009-2018.

Furukawa, K., Katsuno, Y., Urao, T., Yabe, T., Yamada-Okabe, T., Yamada-Okabe, H., Yamagada, Y., Abe, K. & Nakajima, T. (2002). Isolation and functional analysis of a gene, *tcsB,* encoding a transmembrane hybrid-type histidine kinase from *Aspergillus nidulans*. *Appl. Env. Microbiol.* **68**, 5304-5310.

Galbriath, J. & Smith, J. E. (1968). Induction of asexual sporulation in Aspergillus niger in submerged liquid culture. *J Gen Microbioll* **53(suppl)**.

Geißenhöner, A., Sievers, N., Brock, M. & Fischer, R. (2001). *Aspergillus nidulans* DigA, a potential homologue of *Saccharomyces cerevisiae* Pep3(VPS18)(vacuolar sorting), is required for nuclear migration, mitochondrial morphology and polarized growth. *Mol. Genet. Genomics* **266**, 672-685.

Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. & Fink, G. R. (1992). Unipolar cell divisions in the yeast *Saccharomyces cerevisiae* lead to filamentous growth: Regulation by starvation and *RAS*. *Cell* **68**, 1077-1090.

Goffeau A., e. a. (1996). Life with 6000 genes. *Science* **274**, 563-7.

Gould, G. W. & Bell, G. I. (1990). Facilitative glucose transporters: an expanding family. *Trends Biochem. Sci.* **15**, 18-23.

Gustin, M. C., Albertyn, J., Alexander, M. & Davenport, K. (1998). MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **62**, 1264-1300.

Han, D.-M., Han, Y.-J., Chae, K.-S., Jahng, K.-Y. & Lee, Y.-H. (1994). Effects of various carbon sources on the development of *Aspergillus nidulans* with *velA*$^{+}$ or *velA1* allele. *Korean J Mycol* **22**, 332-337.

Han, D.-M., Han, Y.-J., Lee, Y.-H., Jahng, K.-Y., Jahng, S.-H. & Chae, K.-S. (1990). Inhibitory conditions of aseuxual development and their application for the screening of mutants defective in sexual development. *Kor J Mycol* **18**, 225-232.

Han, K. H., Han, K. Y., Yu, J. H., Chae, K. S., Jahng, K. Y. & Han, D. M. (2001). The *nsdD* gene encodes a putative GATA-type transcription factor necessary for sexual development of *Aspergillus nidulans*. *Mol. Microbiol.* **41**, 299-309.

Han, K. H. & Prade, R. A. (2002). Osmotic stress-coupled maintenance of polar growth in *Aspergillus nidulans*. *Mol. Microbiol.* **43**, 1065-1078.

Han, S., Navarro, J., Greve, R. A. & Adams, T. H. (1993). Translational repression of *brlA* expression prevents premature development in *Aspergillus*. *EMBO J.* **12**, 2449-2457.

Hermann, T. E., Kurtz, M. B. & Champe, S. P. (1983). Laccase localized in Hulle cells and cleistothecial primordia of *Aspergillus nidulans*. *J. Bacteriol.* **154**, 955-964.

Herskowitz, I. (1995). MAP Kinase pathways in yeast: For mating and more. *Cell* **80**, 187-197.

Hicks, J. K., Yu, J.-H., Keller, N. P. & Adams, T. H. (1997). *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA Gα protein-dependent signaling pathway. *EMBO J.* **16**, 4916-4923.

Hoffmann, B., Wanke, C., LaPaglia, K. S. & Braus, G. H. (2000). c-Jun and RACK1 homologues regulate a control point for sexual development in *Aspergillus nidulans*. *Mol. Microbiol.* **37**, 28-41.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* **66**, 300-372.

Käfer, E. (1965). Origins of translocations in *Aspergillus nidulans*. *Genetics* **52**, 217-232.

Käfer, E. (1977). Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. *Adv. Genet.* **19**, 33-131.

Karos, M & Fischer, R. (1999). Molecular characterization of HymA, an evolutionarily highly conserved and highly expressed protein of *Aspergillus nidulans*. *Mol. Genet. Genom.* **260**, 510-521.

Kawasaki, L., Sanchez, O., Shiozaki, K. & Aguirre, J. (2002). SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in Aspergillus nidulans. *Mol Microbiol* **45**, 1153-1163.

Kim, H.-S., Han, K.-Y., Kim, K.-J., Han, D.-M., Jahng, K.-Y. & Chae, K.-S. (2002). The *veA* gene activates sexual development in *Aspergillus nidulans*. *Fungal Genet. Biol.* **37**, 72-80.

Kirk, K. E. & Morris, N. R. (1991). The *tubB* α-tubulin gene is essential for sexual development in *Aspergillus nidulans*. *Genes Dev.* **5**, 2014-2023.

Kothe, G. O. & Free, S. J. (1998). The isolation and characterization of *nrc-1* and *nrc-2*, two genes encoding protein kinases that control growth and development in *Neurospora crassa*. *Genetics* **149**, 117-130.

Kruckeberg, A. L. (1996). The hexose transporter family of *Saccharomyces cerevisiae*. *Arch. Microbiol.* **166**, 283-292.

Kübler, E., Mösch, H.-U., Rupp, S. & Lisanti, M. P. (1997). Gpa2p, a G-protein subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. *J Biol Chem* **272**, 20321-20323.

Lee, D. W., Kim, S., Kim, S.-J., Han, D. M., Jahng, K.-Y. & Chae, K.-S. (2001). The *lsdA* gene is necessary for sexual development inhibition by salt in *Aspergillus nidulans*. *Curr. Genet.* **39**, 237-243.

Lengeler, K. B., Davidson, R. C., D'Souza, C., Harashima, T., Shen, W.-C., Wang, P., Pan, X., Waugh, M. & Heitman, J. (2000). Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. Rev.* **64**, 746-785.

Madhani, H. D. & Fink, G. R. (1998). The riddle of MAP kinase signaling specificity. *Trends Genet.* **14**, 151-155.

Madhani, H. D., Styles, C. A. & Fink, G. R. (1997). MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. *Cell* **91**, 673-684.

Marger, M. D. & Saier, M. H., Jr. (1993). A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem Sci* **18**, 13-20.

Martinelli, S. D. & Bainbridge, B. W. (1974). Phenolooxidases of *Aspergillus nidulans*. *Transactions of the British Mycological Society* **63**, 361-370.

Mayorga, M. E. & Gold, S. E. (1999). A MAP kinase encoded by the *ubc3* gene of *Ustilago maydis* is required for filamentous growth and full virulence. *Mol. Microbiol.* **34**, 485-497.

Miller, K. Y., Toennis, T. M., Adams, T. H. & Miller, B. L. (1991). Isolation and transcriptional characterization of a morphological modifier: The *Aspergillus nidulans* stunted (*stuA*) gene. *Mol. Genet. Genomics* **227**, 285-292.

Miller, K. Y., Wu, J. & Miller, B. L. (1992). *StuA* is required for cell pattern formation in *Aspergillus*. *Genes Dev.* **6**, 1770-1782.

Mirabito, P. M., Adams, T. H. & Timberlake, W. E. (1989). Interactions of three sequentially expressed genes control temporal and spatial specificity in Aspergillus development. *Cell* **57**, 859-868.

Mooney, J. L. & Yager, L. N. (1990). Light is required for conidiation in *Aspergillus nidulans*. *Genes Dev.* **4**, 1473-1482.

Mösch, H.-U. (2002). Pseudohyphal growth in yeast. In *Molecular biology of fungal development*, pp. 1-27. Edited by H. D. Osiewacz. New York: Marcel Decker.

Mösch, H.-U. & Fink, G. R. (1997). Dissection of filamentous growth by transposon mutagenesis in *Saccharomyces cerevisiae*. *Genetics* **145**, 671-684.

Müller, P., Aichinger, C., Feldbrügge, M. & Kahmann, R. (1999). The MAP kinase Kpp2 regulates mating and pathogenic develoment in *Ustilago maydis*. *Mol. Microbiol.* **34**, 1007-1017.

Navarro-Garcia, F., Alonso-Monge, R., Rico, H., Pla, J., Sentandreu, R. & Nombela, C. (1998). A role for the MAP kinase gene MKC1 in cell wall construction and morphological transitions in *Candida albicans*. *Microbiology* **144**, 411-424.

O'Rourke, S. M. & Herskowitz, I. (2002). A third osmosensing branch in *Saccharomyces cerevisiae* requires the Msb2 protein and functions in parallel with the Sho1 branch. *Mol. Cell. Biol.* **22**, 4739-4749.

Ozcan, S. & Johnston, M. (1999). Function and regulation of yeat hexose transporters. *Microbiol. Mol. Biol. Rev.* **63,** 554-569.

Pascon, R. C. & Miller, B. L. (2000). Morphogenesis in *Aspergillus nidlans* requires Dopey (DopA), a member of a novel family of leucine zipper-like proteins conserved from yeast to humans. *Mol. Microbiol.* **36**, 1250-1264.

Pöggeler, S. & Kück, U. (2001). Identification of transcriptionally expressed pheromone receptor genes in filamentous ascomycetes. *Gene* **280**, 9-17.

Polacheck, I. & Rosenberger, R. F. (1977). *Aspergillus nidulans* mutant lacking α-1,3-glucan, melanin, and cleistothecia. *J. Bacteriol.* **132**, 650-656.

Pontecorvo, G. & Kafer, E. (1958). Genetic analysis by means of mitotic recombination. *Adv. Genet.* **9**, 71-104.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., MacDonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv Genet* **5**, 141-238.

Prade, R. & Timberlake, W. E. (1993). The *Aspergillus nidulans brlA* regulatory locus consists of two overlapping transcription units that are individually required for conidiophore development. *EMBO J.* **12**, 2439-2447.

Prade, R. A., Ayoubi, P., Krishnan, S., Macwana, S. & Russell, H. (2001). Accumulation of stress and inducer-dependent plant-cell-wall-degrading enzymes during asexual development in *Aspergillus nidulans*. *Genetics* **157**, 957-967.

Reifenberger, E., Freidel, K. & Ciriacy, M. (1995). Identification of novel HXT genes in Saccharomyces cerevisiae reveals the impact of individual hexose transporters on glycolytic flux. *Mol. Microbiol.* **16**, 157-167.

Requena, N., Alberti-Segui, C., Winzenburg, E., Horn, c., Schliwa, M., Philippsen, P., Liese, R. & Fischer, R. (2001). Genetic evidence for a microtubule-destabilizing effect of conventional kinesin and analysis of its consequences for the control of nuclear distribution in *Aspergillus nidulans*. *Mol. Microbiol.* **42**, 121-132

Rosen, S., Yu, J. H. & Adams, T. H. (1999). The *Aspergillus nidulans sfaD* gene encodes a G protein beta subunit that is required for normal growth and repression of sporulation. *EMBO J* **18**, 5592-5600.

Sabbagh, W., Flatauer, L. J., Bardwell, A. J. & Bardwell, L. (2001). Specificity of MAP kinase signaling in yeast differentiation involves transient versus sustained MAPK activation. *Mol. Cell* **8**, 683-691.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sambrook, J. & Russel, D. W. (1999). *Molecular Cloning: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Scherer, M. (2001). Molekulare Analyse der sexuellen Entwicklung von Aspergillus nidulans. Dissertation. In *Fachbereich Biologie*. Marburg, Germany: Philipps-Universität.

Scherer, M. & Fischer, R. (1998). Purification and characterization of laccase II of *Aspergillus nidulans*. *Arch. Microbiol.* **170**, 78-84.

Scherer, M., Wei, H. & Fischer, R. (2002). Aspergillus nidulans catalase-peroxidase, CpeA, is upregulated during sexual development through the APSES transcription factor StuA.*Eukaryotic Cell.* **1(5)**, 725-35.

Schier, N., Liese, R. & Fischer, R. (2001). A pcl-like cyclin of *Aspergillus nidulans* is transcriptionally activated by developmental regulators and is involved in sporulation. *Mol. Cell. Biol.* **21,** 4075-4088.

Schramek, H. (2002). MAP kinases: from intracellular signals to physiology and disease. *News Physiol.* **17**, 62-70.

Stork, P. J. & Schmitt, J. M. (2002). Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell. Biol.* **12**, 258-266.

Stringer, M.A., Dean, R.A., Sewall, T.C. & Timberlake, W.E. (1991). *Rodletless*, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Gene and Develop.* **5,** 1161-1171

Timberlake, W. E. (1990). Molecular genetics of *Aspergillus* development. *Ann. Rev. Genet.* **24**, 5-36.

Upshall, A. (1981). Naturally occurring dipoid isolates of Aspergillus nidulans. *J Gen Microbiol Biol* **122**, 7-10.

Vallim, M. A., Miller, K. Y. & Miller, B. L. (2000). *Aspergillus* SteA (Sterile12-like) is a homeodomain-C_2/H_2-Zn^{+2} finger transcription factor required for sexual reproduction. *Mol. Microbiol.* **36**, 290-301.

Varma A., Singh B. B., Karnani N., Lichtenberg-Frate H., Hofer M., Magee B. B. & R., P. (2000). Molecular cloning and functional characterisation of a glucose transporter, CaHGT1, of Candida albicans. *FEMS Microbiol Lett* **182**, 15-21.

Vienken, K. (2002). Der Zn(II)2Cys6 binukleäre Transkriptionsfaktor ProA reguliert Entwicklungsentscheidungen in *Aspergillus nidulans*. Diplomarbeit. *Fachbereich Biologie - Mikrobiologie* -. Marburg: Philipps-Universität Marburg.

Vienken, K., Scherer, M. and Fischer, R. (2003). The Zn(II)2Cys6 transcription factor RosA (repressor of sexual development) inhibits sexual development under inappropriate condition in the filamentous fungus *Aspergillus nidulans*. *Submitted*

Waring, R. B., May, G. S. & Morris, N. R. (1989). Characterization of an inducible expression system in *Aspergillus nidulans* using *alcA* and tubulin coding genes. *Gene* **79**, 119-130.

Weber, R. (2002). Die Funktion von HxtA im sexuellen Zyklus von *Aspergillus nidulans*. Diplomarbeit. *Fachbereich Biologie - Mikrobiologie* -. Marburg: Philipps-Universität Marburg.

Wei, H., Requena, N. and Fischer, R. (2003). The MAPKK-kinase SteC regulates conidiophore morphology and is essential for hyphal fusion and sexual development in the homothallic fungus *Aspergillus nidulans. Molecular Microbiology.* 47(6), 1577-1588

Wei, H., Scherer, M., Singh, A., Liese, R. & Fischer, R. (2001). *Aspergillus nidulans* alpha-1,3 glucanase (mutanase), *mutA*, is expressed during sexual development and mobilises mutan. *Fungal Genet. Biol.* **34**, 217-227.

Weierstall, T., Cornelis, P. H. & Boles, E. (1999). Cloning and characterizazion of three genes (SUT1-3) encoding glucose transporetrs of the yeast Pichia stipitis. *Mol. Microbiol.* **31**, 871-883.

Wesolowski-Louvel, M., Goffrini, P., Ferrero, I. & Fukuhara, H. (1992). Glucose transport in the yeast *Kluyveromyces lactis*. I. Properties of an inducible low-affinity glucose transporetr gene. *Mol. Gen. Genet.* **233**, 89-96.

Wieser, J. & Adams, T. H. (1995). *flbD* encodes a myb-like DNA-binding protein that coordinates initiation of *Aspergillus nidulans* conidiophore development. *Genes Dev.* **9**, 491-502.

Wieser, J., Lee, B. N., Fondon III, J. W. & Adams, T. H. (1994). Genetic requirements for initiating asexual development in *Aspergillus nidulans*. *Curr. Genet.* **27**, 62-69.

Wieser, J., Yu, J. H. & Adams, T. H. (1997). Dominant mutations affecting both sporulation and sterigmatocystin biosynthesis in *Aspergillus nidulans*. *Curr Genet* **32**, 218-224.

Wieczorke, R., Krampe, S., Weierstall, T., et al. (1999). Concrurent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett* **464,** 123-128.

Wood V, e. a. (2002). The genome sequence of Schizosaccharomyces pombe. *Nature* **415**, 871-880.

Wu, J. & Miller, B. L. (1997). *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating *stunted* gene expression. *Mol. Cell. Biol.* **17**, 6191-6201.

Xu, J. R. (2000). MAP kinases in fungal pathogens. *Fungal Genet. Biol.* **31**, 137-152.

Yarden, O., Plamann, M., Ebbole, D. J. & Yanofsky, C. (1992). *cot-1*, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. *EMBO J.* **11**, 2159-2166.

Yelton, M. M., Hamer, J. E. & Timberlake, W. E. (1984). Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc. Natl. Acad. Sci. USA* **81**, 1470-1474.

Yu, J.-H., Wieser, J. & Adams, T. H. (1996). The Aspergillus FlbA RGS domain protein antagonizes G-protein signaling to block proliferation and allow development. *EMBO J.* **15**, 5184-5190.

Zonneveld, B. J. M. (1971). Biochemical analysis of the cell wall of *Aspergillus nidulans*. *Biochim. Biophys. Acta* **249**, 506-514.

Zonneveld, B. J. M. (1972a). Morphogenesis in *Aspergillus nidulans*. The significance of α-1,3 glucan of the cell wall and α-1,3 glucanase for cleistothecium development. *Biochim. Biophys. Acta* **273**, 174-187.

Zonneveld, B. J. M. (1972b). A new type of enzyme, an exo-splitting α-1,3 glucanase from non-induced cultures of *Aspergillus nidulans*. *Biochim. Biophys. Acta* **258**, 541-547.

Zonneveld, B. J. M. (1973). Inhibitory effect of 2-deoxy glucose on cell wall α-1,3-glucan synthesis and cleistothecium development in *Aspergillus nidulans*. *Dev. Biol.* **34**, 1-8.

Zonneveld, B. J. M. (1974). α-1,3 glucan synthesis is correlated with α-1,3 glucanase synthesis, conidiation and fructification in morphogenetic mutants of *Aspergillus nidulans*. *J. Gen. Microbiol.* **81**, 445-451.

Zonneveld, B. J. M. (1975). Sexual differentiation in *Aspergillus nidulans,* The requirement for manganese and the correlation between phospoglucomutase and the synthesis of reserve material. *Arch Microbiol* **105**, 105-108.

Zonneveld, B. J. M. (1977). Biochemistry and ultrastructure of sexual development in *Aspergillus*. In *Genetics and Physiology of Aspergillus*, pp. 58-80. Edited by J. E. Smith & J. A. Pateman. London: Academic Press.

Fungal Genetics and Biology **34**, 217–227 (2001)
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Aspergillus nidulans α-1,3 Glucanase (Mutanase), *mutA*, Is Expressed during Sexual Development and Mobilizes Mutan

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Wei, H., Scherer, M., Singh, A., Liese, R., and Fischer, R. 2001. *Aspergillus nidulans* α-1,3 glucanase (mutanase), *mutA*, is expressed during sexual development and mobilizes mutan. *Fungal Genetics and Biology* **34**, 217–227. We established a subtractive cDNA library of *Aspergillus nidulans* to identify differentially expressed genes during sexual development. One of the clones displayed homology to fungal α-1,3 glucanases (mutanase). Since α-1,3 glucan is considered the main reserve material accumulated during vegetative growth as a cell wall component and consumed during sexual development, we analyzed this gene in detail. The gene, *mutA*, is disrupted by three introns and encodes a putative protein of 48 kDa molecular mass with a signal peptide for secretion at the N terminus. The deduced protein displays amino acids 24–42% identical to mutanases of other fungi. A proposed mutan binding domain characterized in, e.g., *Penicillium* is not present in *A. nidulans*. Mutanase transcript and GFP reporter analysis in *A. nidulans* revealed specific induction of the gene during sexual development in Hülle cells. To study the role of *mutA* during sexual differentiation, we constructed a *mutA* deletion strain. Although degradation of mutan was affected in this strain, it was still able to form cleistothecia at a number similar to that of wildtype. These results suggest that additional carbon sources are available during sexual development.

Index Descriptors: spore formation; mutanase; fungal development; fungal cell wall; fruiting body (cleistothecium); Hülle cell; suppressive subtractive hybridization.

The filamentous fungus *Aspergillus nidulans* is able to reproduce with mitotically derived conidiospores and by meiotic ascospores (Adams *et al.*, 1998). Both spore forms are generated at or in morphologically differentiated structures called conidiophores and cleistothecia, respectively (Fischer, 2001). Whereas the developmental program of conidiophore formation is well studied, cleistothecium differentiation is only poorly understood. *A. nidulans* is especially attractive for the analysis of fruiting body formation because it is a homothallic fungus and does not require a mating partner to initiate the developmental program.

The first sign of the differentiation process is that some hyphae grow out at the base of a conidiophore, branch, and form a clump of hyphae. Some of the filaments swell subapically or apically to form specialized cell types, the Hülle cells. These are surrounded by very thick cell walls. They are produced in large numbers and cover the young fruiting bodies. Within the hyphal mass asci, which contain

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Aspergillus nidulans Catalase-Peroxidase Gene (*cpeA*) Is Transcriptionally Induced during Sexual Development through the Transcription Factor StuA

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Catalases, peroxidases, and catalase-peroxidases are important enzymes to cope with reactive oxygen species in pro- and eukaryotic cells. In the filamentous fungus *Aspergillus nidulans* three monofunctional catalases have been described, and a fourth catalase activity was observed in native polyacrylamide gels. The latter activity is probably due to the bifunctional enzyme catalase-peroxidase, which we characterized here. The gene, named *cpeA*, encodes an 81-kDa polypeptide with a conserved motif for heme coordination. The enzyme comprises of two similar domains, suggesting gene duplication and fusion during evolution. The first 439 amino acids share 22% identical residues with the C terminus. Homologous proteins are found in several prokaryotes, such as *Escherichia coli* and *Mycobacterium tuberculosis* (both with 61% identity). In fungi the enzyme has been noted in *Penicillium simplicissimum*, *Septoria tritici*, and *Neurospora crassa* (69% identical amino acids) but is absent from *Saccharomyces cerevisiae*. Expression analysis in *A. nidulans* revealed that the gene is transcriptionally induced upon carbon starvation and during sexual development, but starvation is not sufficient to reach high levels of the transcript during development. Besides transcriptional activation, we present evidence for post-transcriptional regulation. A green fluorescent protein fusion protein localized to the cytoplasm of Hülle cells. The Hülle cell-specific expression was dependent on the developmental regulator StuA, suggesting an activating function of this helix-loop-helix transcription factor.

Oxidative stress and the occurrence of reactive oxygen species is common to aerobically living organisms and might be deleterious for living cells (10, 18). Reactive oxygen species are generated during normal cell metabolism and comprise superoxide, hydroxyl radicals, hydrogen peroxide, and singlet oxygen. All aerobically living organisms employ one or several systems to cope with these toxic substances. Catalases and peroxidases are most commonly used to transform the harmful oxygen compound H_2O_2 into harmless products. Catalases are heme-containing enzymes, which convert H_2O_2 into oxygen and water. Peroxidases are heme-containing enzymes as well and inactivate H_2O_2 by reducing it to water. In addition to heme-containing catalases and peroxidases, nonheme varieties of these enzymes exist. Different cellular substrates can serve as electron donors for this reaction. Frequently, organisms use different isozymes, which are expressed simultaneously or under developmental-stage- and environment-specific conditions (26, 35).

One good example for the employment of several catalases and their differential regulation during the life cycle is the filamentous fungus *Aspergillus nidulans* (15). *A. nidulans* is able to grow as vegetative hyphae but then undergoes two developmental programs. After 20 h of vegetative growth it can enter an asexual reproductive pathway in which it generates thousands of single-cell, haploid conidiospores. In addition, it is able to reproduce itself with very durable sexually derived ascospores (1). Both spore types are produced at or in special morphological structures, called conidiophores or cleistothecia, respectively. The conidiophore consists of four different cell types—a stalk, metulae, phialides, and conidia—and grows away from the agar surface into the air. The asexual developmental pathway is very well characterized at the molecular level (1) and is triggered by a central cascade of transcriptional activators (3, 22). In an effort to characterize differentially expressed genes during asexual development, a catalase gene (*catA*) was discovered (20) that is transcriptionally and post-transcriptionally regulated, and the protein accumulated in conidiospores (19). Using the *catA* sequence, a second catalase, *catB*, was isolated (16). This gene is developmentally induced during conidiophore formation, but the transcript is almost absent in conidiospores. The *catB* expression, like that of *catA*, also responds to different stress conditions (6, 16). Finally, Kawasaki and Aguirre, taking advantage of the genomic sequencing project at Cereon Genomics LLC (Cambridge, Mass.), identified a third catalase gene, designated *catC* (15). The protein resides in peroxisomes and is constitutively expressed. Interestingly, mutations in a single gene or in all catalase genes did not have any detectable vegetative or developmental phenotype, suggesting the presence of more isozymes. Indeed, in native polyacryl amide gels a fourth catalase activity, *catD*, was observed (15). We have identified the corresponding gene and found that it is a catalase-peroxidase. The expression of the gene is transcriptionally and translationally regulated upon carbon starvation and during sexual development. One important regulator is the transcription factor StuA.

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The MAPKK kinase SteC regulates conidiophore morphology and is essential for heterokaryon formation and sexual development in the homothallic fungus *Aspergillus nidulans*

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Summary

Environmental signals can be transduced into intracellular responses by the action of MAP kinase cascades. Sequential phosphorylation results in the transient activation of a MAP kinase, which in turn activates certain transcription factors and thus a set of pathway-specific genes. Many steps in this cascade are conserved, and homologues have been discovered from yeast to human. We have characterized the MAPKK kinase, SteC, a homologue of *Saccharomyces cerevisiae* Ste11, in the filamentous fungus *Aspergillus nidulans*. The 886-amino-acid-long protein shares the highest similarity to *Neurospora crassa* Nrc-1. Deletion of the gene in *A. nidulans* results in a slower growth rate, the formation of more branched hyphae, altered conidiophore morphology, an inhibition of heterokaryon formation and a block of cleistothecium development. The gene is transcriptionally activated during asexual development and controls the phosphorylation of two putative MAP kinases.

Introduction

Mitogen-activated protein kinases (MAP kinases) are ubiquitous among eukaryotes. MAP kinases are components of MAP kinase cascades, which are major signalling modules by which cells transduce extracellular cues into intracellular responses (Gustin *et al*., 1998; Stork and Schmitt, 2002). Originally, they were described as protein kinases, which were transiently activated by a variety of mitogens, including insulin or growth factors, and are thus implicated in cell proliferation and regulation of the cell cycle. Misregulation in animal cells leads to inappropriate activation of cell division and may result in the development of cancer (Schramek, 2002). The basic mechanism of signal transduction appears to be very similar in different MAP kinase cascades, namely a sequential activation of protein kinases upon external stimulation with a signal. One early kinase after signal recognition is a MAP kinase kinase kinase, which phosphorylates a MAP kinase kinase at two amino acid residues. The latter kinase in turn activates a MAP kinase, again by dual phosphorylation. The phosphorylated MAP kinase triggers the activity of transcription factors, and thus the external signal is transmitted from the surface of the cell into the nucleus.

The evolutionary conservation of MAP kinase signalling pathways allows the use of lower eukaryotes such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* as models to unravel the molecular and biochemical functions of the components (Herskowitz, 1995). In *S. cerevisiae*, at least five different MAP kinase cascades exist, which differ in the signals that are perceived and transmitted, in the activation of the specific MAP kinase and, ultimately, a specific transcription factor (Gustin *et al*., 1998; Hohmann, 2002). The cascades are involved in mating, nutrient sensing and pseudohyphal growth, osmoregulation and stress adaptation, cell integrity and ascospore formation. Thus, the cellular responses are as diverse as the induction of mating upon pheromone perception and the synthesis of compatible solutes upon osmotic stress. Nevertheless, some signalling molecules are used in different cascades. Likewise, the MAPKK kinase Ste11 is involved in mating, pseudohyphal growth and osmoregulation. The regulation of the specificity of each cascade and the prevention of cross-talk between them are one important and largely unsolved questions (Sabbagh *et al*., 2001). In plant or human pathogenic fungi, MAP kinase cascades are involved in triggering the pathogenic programme and adaptation to the host-specific environmental conditions (Mayorga and Gold, 1999; Müller *et al*., 1999; Lengeler *et al*., 2000; Xu, 2000; Mey *et al*., 2002).

MAP kinase cascades are involved in major developmental transitions in the life cycle of the unicellular fungus

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